A model for isolated rat liver perfusion in normo- and hypothermia. Description and application in liver preservation research

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ISBN 90-9002987-7

RIJKSUNIVERSITEIT TE GRONINGEN

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PROEFSCHRIFT

ter verkrijging van het doctoraat in de Geneeskunde
aan de Rijksuniversiteit te Groningen
op gezag van de
Rector Magnificus Dr. L.J. Engels
in het openbaar te verdedigen op woensdag 13 september 1989
des namiddags te 4.00 uur

door

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geboren te Drachten

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Prof. Dr. M.J. Hardonk
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Prof. Dr. G.L. Scherphof

De verschijning van dit proefschrift werd mede mogelijk gemaakt door financiele steun van de J.K. de Cock stichting.

Voor Jolien,

Julie en

Sarah

CONTENTS

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ALT	alanine transaminase
AST	aspartate transaminase
c_x	concentration of the substance x
FAD	flavin adenine dinucleotide
FADH	reduced FAD
ID	inner diameter
IU	international units (enzyme activity)
K'	apparent equilibrium constant
M	molar (mole/liter)
Md	median
n	(sample) number
NAD	nicotinamide adenine dinucleotide
NADH	reduced NAD
OD	outer diameter
P	hydrostatic pressure/partial pressure/gas tension in a solution
Px	partial pressure of the gas x/ tension of the gas x in a solution
$\dot{Q}$	fluid volume per unit of time (flow)
R	resistance
R.Q.	respiratory quotient
T	temperature
V	gas volume
$\dot{V}$	gas volume per unit of time

A force de frapper à coups
redoublés sur la même porte,
elle finit toujours par s'ouvrier.
Ou alors c'est une porte voisine,
qu'on n'avait pas vue, qui
s'entrebâille, et c'est
encore plus beau.

Michel Tournier
Le Roi des Aulnes

GENERAL INTRODUCTION

Progress in clinical liver preservation depends strongly on the extension of the period during which the liver can be preserved before being implanted in the acceptor's body (1,2). Progress is closely related to increase of the knowledge on the way biological processes are influenced by 1. disconnecting the liver from its natural circulation, thus subjecting it to a period of warm ischemia, and by 2. changing environmental conditions (i.e. lowering the temperature) in an attempt to reduce the detrimental effects of ischemia (3,4).

During the last three decades, a large number of papers has been published on preservation technology and on the effect of cooling on fundamental (sub) cellular processes. However, the amount of data is too diverse to become integrated and applied directly in an usable method of liver preservation which enables longterm preservation of the liver without the risk of disrupting its functional and morphological integrity.

Integration of the data is difficult for two reasons. Firstly, studies in organ preservation mostly concern the preservation of organs at a temperature of 10°C or lower. This leaves the whole temperature range from 10 to 37°C beyond consideration even though it has been suggested that preservation temperatures above 10°C may eventually prove to be more suitable (4). Secondly, many data on fundamental biological processes have been brought about primarily by studies on the (sub) cellular level, whereas organ preservation concerns the organ as a whole. Therefore, successful research in liver preservation requires a model in which the effects of ischaemia and varying environmental conditions (i.e. normo- and hypothermia) on the isolated liver

can be studied.

This thesis deals primarily with the development and characterization of a perfusion model for the study of the functioning of the isolated rat liver in normo- and hypothermia. This is not the first model: Isolated liver perfusion has been performed already in the 19th century, and the first isolated rat liver perfusion model dates from 1942 (5) However, none of the models is suitable for hypothermic perfusion and rapid cooling and (re-) warming of the perfused rat liver.

OUTLINE

This thesis is devided into three sections. In the first section (chapter 1) an overview is given of the literature on the effect of hypothermia on the functioning of biological systems, of the preservation methods, and of the preservation media that have been used in the past decades. This extensive overview is given to provide a general background for the pricipal subject of this thesis, i.e. the development of a simple model for perfusion of the isolated rat liver in normo- and hypothermia.
In section two (chapters 2 and 3) the perfusion apparatus, the technique of hepatectomy and the materials and methods are described. The rat liver model was chosen because of its simplicity, because it offers the possibility to perform relatively large studies on a whole organ, and because of its cost effectiveness.
In section three the model is further characterized with regard to its applicability in studying morphology and metabolic,

synthetic and perfusion dynamic characteristics in normo- and hypothermia (chapters 4,5,6,7,8) of livers, whether or not treated with normothermic anoxia prior to perfusion. Furthermore, the model is used to study the cytoprotective effect of a calcium entry blocker on anoxic damaged livers (chapter 9). Finally, the application of the perfusion apparatus as a recirculating system is demonstrated in chapter 10. This chapter concerns the influence of bile acid infusion on bile production of the isolated perfused rat liver.

SECTION I

REVIEW OF LITERATURE

CHAPTER 1

PRESERVATION TECHNOLOGY: TEMPERATURE, PRESERVATION MEDIA AND PRESERVATION METHODS

INTRODUCTION

The development of new methods of liver preservation demands answers to questions concerning the functioning of isolated livers in normo- and hypothermia, the preservation media, and the preservation equipment.

Hypothermia is considered the single most important factor in organ preservation. However, it has been recognized that cooling may produce also several adverse effects, such as cellular swelling and depletion of high energy nucleotides, that possibly limit the maximum preservation time of isolated organs (6).

Many papers have been published the past three decades on the effect of cooling on structure and functioning of biological membranes, and on fundamental physical, biochemical and physiological processes in biological systems. Most of these papers concerned studies that were done at cellular and subcellular level. Several of them are reviewed in the present chapter.

To preserve isolated organs, one can choose one of two approaches. The first is to mimic the normal situation by replacing the circulation by a pump oxygenator system, and to perfuse the organ continuously.The second is to flush the organ followed by storage at a low temperature. In both approaches fluid either to perfuse or to flush and cool the organ is needed. The perfusates that have been developed can be divided in two main groups: natural and artificial. Within these groups further subdivisions can be made. To cope with the basic problems of organ preservation -i.e. the occurrence of oedema, increasing

vascular resistance and cell damage- attempts have been made to improve the quality of the preservation media by improving electrolyte balance and/or by adding substances that provide oncotic pressure, adjust pH, meet the metabolic needs of the tissue, or that provide special properties such as oxygen carriage, sterility, vasomediation, cell protection and metabolic inhibition.

Several natural and artificial preservation media and additives are discussed in this chapter.

Furthermore, important preservation techniques are reviewed.

TEMPERATURE

TERMINOLOGY

The terminology to describe the reduction of the temperature of the body as a whole and of isolated organs, tissues or cells varies and is confusing. Cooling, overall cooling, over cooling, supercooling, and freezing are terms which refer to the mechanism of action. Artificial hibernation, hibernation, suspended animation, "Scheintod", and hypothermia are used to describe a state of reduced body, organ, tissue or cellular temperature. In this thesis, only the terms cooling and hypothermia will be used. Cooling implies the presence of an external factor, that will only reduce the temperature of the intact body of a warmblooded (homeothermic) animal, when its thermoregulatory mechanisms can no longer cope with the thermal stress. It is evident, that isolated organs, tissues and cells are very susceptible to thermal stress from their environment, because they are no longer part of thermoregulatory circuits.

Hypothermia is a state in which the central body temperature of the homeotherm has fallen below 36°C, the physiological underlimit of normothermia (7). The standard homeothermia is that of homeotherms. Nevertheless, the term hypothermia also applies to poikilotherms. Because the hibernal sleep state is a normothermic state, adapted to a low environmental temperature, hypothermia does not apply to hibernators. They only pass through a phase of true hypothermia before falling asleep (8).
Five stages of hypothermia have been defined between 0 and 36°C: augmented (33-32°C), moderate (30-28°C), moderately deep (25°C), deep (20°C), and profound hypothermia (10°C) (9).
In summary, with regard to the preservation of isolated organs, cooling is the mechanism that results in a state of augmented, moderate, moderately deep, deep or profound hypothermia.

THE STRUCTURE AND FUNCTION OF MEMBRANES

In the classical concept of Singer and Nicolson, membranes are considered as "fluid mosaic" arrangements of lipids and proteins (10). It is assumed that membrane characteristics and membrane functions are regulated by the interaction of the lipids and the proteins.
In this respect, a key property of the lipids is their "fluidity", which can be defined as the relative motional freedom of the lipid molecules (11,12). The fluidity of membranes and, consequently, the functioning of membrane proteins are influenced by many factors including temperature.
In model bilayer membranes, composed of a single species of

lipid, characteristic narrow temperature ranges ("phase transition temperature":T_c), can be demonstrated. In these temperature ranges the physical state of the lipid changes from a more solid and ordered ("gel"), to a more fluid and disordered ("liquid crystalline") phase, or the other way around. In natural membranes, consisting of a complex mixture of phospholipids and containing sometimes relatively large quantities of cholesterol, the lipid thermotropic phase transitions are broad (13,14). Depending on the method of quantifying membrane fluidity, the lipid thermotropic phase transition temperatures of rat hepatocyte plasma membranes have been shown to vary from 17.5 to 31°C (14,15,16,17,18,19).

Thermotropic transitions may also lead to lateral phase separation between lipids and between lipid-phases or proteins. This gives rise to the formation of discrete regions of crystalline, gel state lipid and clusters of protein aggregates. Transitions of this kind have been demonstrated for human erythrocyte membranes (20).

ENZYME CATALYZED REACTIONS

Enzyme catalyzed reactions are influenced by temperature in two ways: i.e. by the effect on the number of activated substrate molecules and by the effect on enzyme activity ("thermal inactivation").

It has been shown that the activity of a great number of enzymes in hypothermia is less than would be predicted by extrapolating from their activities at higher temperatures (21,22,23). These

changes cannot only be ascribed to changes in the fluidity of membranes in which a great number of enzymes is embedded (22). There are studies which indicate that also changes in the protein conformation of enzymes may cause a non-linear relation between enzyme activity and temperature (23).

ENERGY METABOLISM

Most of the cell's energy consuming reactions utilize adenosine triphosphate (ATP) as a universal source of energy. ATP is resynthesized during glycolysis and oxidative phosphorylation. Glycolysis takes place in the cytosol. It involves the transduction of energy from oxidation reactions, leading to the formation of ATP from adenosine diphosphate (ADP) and phosphate (Pi). In this process, which is called substrate level phosphorylation, glucose is oxidized to pyruvate.
The glycolytic rate is controlled by the availability of nicotinamide dinucleotide (NAD). For the continuation of glycolysis, reduced NAD (NADH) must be reoxidized continuously. Under aerobic conditions, this reoxidation is accomplished by shuttling NADH to the mitochondrial matrix, where it is reoxidized by the respiratory chain (24).
Oxidative phosphorylation takes place in the mitochondrion. In this process the remainder of the bond energy in glucose and the major part of the bond energy in fatty acids is made available, thus accounting for the major part of the cell's ATP synthesis. Acetyl-CoA, formed from pyruvate or by beta-oxidation of fatty acids, is oxydized to CO_2 in the Krebs cycle. This involves a

number of NAD or FAD (flavin-adenine-nucleotide) coupled dehydrogenases, located in the mitochondrial matrix. The reduced co-enzymes NADH and $FADH_2$, formed in the Krebs cycle, are reoxidized in the electron transport chain, which is embedded in the mitochondrial inner membrane. Because NADH and FADH have highly negative redox potentials, they easily donate electrons to the oxidized cytochromes of the electron transport chain. The energy, which is released in the reoxidation of NADH and FADH, is utilized to produce ATP from ADP and Pi.

Many investigators have tried to elucidate this mechanism of energy coupling, i.e. the coupling of oxidative and phosphorylating reactions (25,26).

Current theory proposes that the respiratory chain dissociates hydrogen atoms from NADH and FADH to form protons (H^+) and electrons (e^-). The protons are released from the mitochondria, and the electrons pass through the respiratory chain. Ultimately oxygen accepts the electrons and protons to form H_2O.

The flux of protons to the outside of the mitochondria results in a pH gradient -the inside becoming alkaline- and an electrical potential across the membrane -the inside being negative. Ultimately this flux arrests because of the back pressure, which the proton gradient produces itself. At that point the electron transport and the (re-) oxidation of NADH and FADH slows down or eventually stops. Consequently, the flow of substrate through the Krebs cycle will stop because NAD is no longer available when the oxidation of NADH is arrested.

This inhibitory effect of the proton gradient is relieved by the back flow of protons into the mitochondrial matrix through

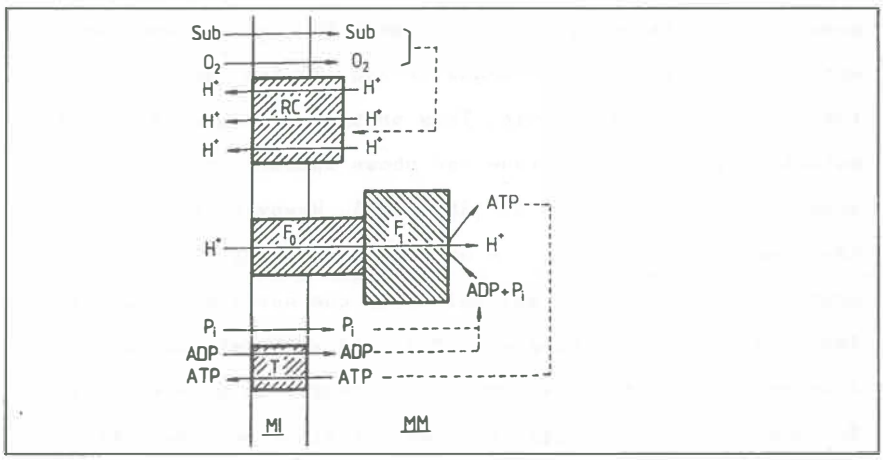


Fig.1-1: The mechanism of energy coupling: As long as ADP and Pi are available to be converted to ATP, protons (H+) flow back to the mitochondrial matrix (MM) and electron transport, re-oxidation of reduced co-enzymes and the flux of substrate (SUB) through the Krebs cycle will continue. Fo=specific proton channels; F1=ATP-ase; RC=respiratory chain; MI=mitochondrial inner mebrane; T=translocase.

specific proton channels (Fo). These are structurally and functionally coupled to the enzyme ATPase (F1), which catalyzes the resynthesis of ATP from ADP and Pi during the influx of protons through the channels. Protons flow back to the mitochondrial matrix at the "ATPase sites" as long as ATP is being formed, i.e. ADP and Pi are available.

Thus, the mechanism of energy coupling (i.e. oxidative phosphorylation) is controlled by ADP: As long as ADP and Pi are available to be converted to ATP, protons can flow back to the mitochondrial matrix and electron transport, reoxidation of reduced coenzymes, and the flux of substrate through the Krebs cycle will continue (Fig. 1-1).

ADP, formed outside the mitochondria during energy requiring

processes, is taken up by mitochondria in a one-to-one exchange with mitochondrial ATP by means of the adenine nucleotide translocase carrier protein. This protein is embedded in the mitochondrial inner membrane and shows specific affinity for adenine nucleotides (ATP,ADP,AMP) (24). However, because of the transmembrane potential, the exchange of matrix ATP^{-4} for cytosolic ADP^{-3} or the equivalent of the outflow of an anion is favoured. Cytosolic phosphate (Pi) enters the mitochondria independently, not driven by the transmembrane potential (24,25). Because ADP is the trigger for the oxidative phosphorylation, energy coupling in the intact cell depends on the rate of exchange of matrix ATP for cytosolic ADP, i.e. on the activity of the translocase protein. Normally, the rate of adenine nucleotide translocase is not a limiting factor for the process of energy coupling, although the adenine nucleotide translocation has only little or no catalytic capacity in excess (24).

The influx of Pi into the mitochondria in exchange for OH^- ions occurs in about a 20-fold higher rate than the processes, controlled by adenine nucleotide translocase.

Thus, the entry of ADP into the mitochondrial matrix, i.e. the translocase rate, is a critical factor in energy coupling.

Hypothermia may influence the energy metabolism by altering the transport capacity and the preference for specific substrates, by changing the transport capacity for adenine nucleotides, and by affecting the catalytic properties of enzymes of the intermediary metabolism.

It has been shown that the preference for substrates may change with the temperature. At 38°C well oxygenated kidney cortex

slices metabolize glucose, fatty acids (in particular longer chain fatty acids), ketone bodies and amino acids. At 10°C, however, these slices predominantly consume short-chain fatty acids (26,27).

The causes of the defective carbohydrate metabolism in hypothermia are not entirely clear. Both, enzyme inhibition (28) and failure of the transmembrane transport of glucose (29) may have some effect.

In hypothermia, the uptake of glucose is reduced, although it has been demonstrated that glucose and the eight-carbon fatty acid caprylic acid are taken up by the hypothermically perfused kidney at comparable rates (30). Glucose was converted to lactate under these conditions, while the fatty acid was predominantly oxidized to CO_2. At this point also the partial oxygen tension (P_{O2}) of the perfusate may be of importance, since the amount of oxidized caprylic acid increased when the oxygen tension in the perfusate was raised (31). Furthermore, it has been demonstrated that at high oxygen tensions (about 600 mmHg/80 kPa)the oxidation of fatty acid (caprilyc acid) delivers the greater part of the cell's energy, and that both, glucose utilization and lactate production (i.e. glycolysis) are reduced. At lower oxygen tensions (about 150 mmHg/20 kPa), however, glycolysis appeared to be the main supplier of energy (4). Therefore, it is tempting to conclude that hypothermia alters not only the preference for substrate - i.e. fatty acid combustion being preferred to glucose oxidation - but also the balance between aerobic and anaerobic metabolism at a given oxygen tension.

The transport of adenine nucleotides across the mitochondrial membrane may also be affected in hypothermia. Because there is only little spare catalytic capacity for adenine nucleotide translocation, and because nucleotide translocation shows a break point in the Arrhenius plot at 18°C (24) there is a risk that below 18°C the exchange of mitochondrial ATP for cytosolic ADP is hampered, irrespective of the fact that oxidative phosphorylation rates are adequate to synthesize ATP. This situation results in the deterioration of cell function, unless, by cooling, the energy utilizing rates are depressed to the same extent as the translocase activity.

Another possible consequence of the reduced adenine nucleotide translocase activity is the loss of nucleotides from the cell, which depends on dephosphorylation reaction rates, and the high permeability of the cellular membrane for the resulting nucleosides. In this, an important role is played by the enzyme 5'-nucleotidase, which catalyzes the conversion of adenosine-mono-phosphate (AMP) to adenosine. This reaction is a function of the cytosolic AMP level, while it is inhibited by ADP and ATP (24). As to the effect of hypothermia on this reaction, it is noteworthy that the activity of 5'-nucleotidase shows a break point in the Arrhenius plot at 17°C (14). The cytosolic level of AMP may increase as a result of the conversion of ADP, which will accumulate when the translocase activity is reduced in hypothermia; two molecules of ADP are converted to AMP and ATP, one molecule each. This reaction is catalyzed by adenylate kinase. In addition, AMP may originate from adenosine in a reaction, catalyzed by adenosine kinase (24). In organ

preservation, an additional factor to the depletion of adenine nucleotides from the cells is ischaemia which leads to a rapid accumulation of AMP (31).

Adenosine, when not converted to AMP, is very rapidly deaminated to inosine. This has to be transformed to hypoxanthine before conversion to high-energy phosphate compounds is possible again. The uptake of hypoxanthine by the cell and the formation of ATP from it is a very slow process as compared to the "salvage reaction" of adenosine (24). The latter reaction is, however, likely to slow down under preservation conditions since it requires ATP.

Finally, there are indications that the process of oxidative phosphorylation itself may become defective in hypothermia. In this respect, it has been, for instance, demonstrated that deep hypothermia inhibits the succinate oxidase system (32).

TRANSMEMBRANE TRANSPORT

Hypothermia causes a reduction in passive transmembrane transport of particles (solute as well as solvent). In this, it is noteworthy that membrane fluidity is a determinant of the passive permeability of membranes (33,34,35). Active transport mechanisms ("membrane pumps") -i.e. transport across membranes other than by diffusion or osmosis, mostly against a transmembrane concentration gradient and requiring the expenditure of energy by the cell- exists for many substances, e.g. ions (Na^+, K^+, Ca^{2+}, H^+), glucose and amino acids.Hypothermia may influence these mechanisms in two ways. Firstly, by impairing the generation of

ATP. Secondly by preventing the active transport mechanism to utilize ATP. It has, for instance, been shown that in profound hypothermia Na^+/K^+-ATPase activity in most tissues is small (36), causing the sodium pump to stop. Thus, even when ample supplies of ATP are present (which is, however, theoretically unlikely) active transport mechanisms are not able to utilize the energy in (profound) hypothermia.

CIRCULATION

Hypothermia induces profound alterations in the haemodynamics, and bloodflow distribution of the intact animal body. As the body temperature falls, the heart rate slows progressively (37,38,39,40,41) along with a decline in cardiac output (41,42,43,44) and blood pressure (41,43,45).

The microcirculation shows significant slowing of the red cell flow below 25°C (46). Furthermore, lymph flow appears to follow the same pattern as the heart rate and the arterial pressure during cooling. There are also indications that the reduction in the lymph flow is related to the shut down of microcirculation, resulting in the reduction of the capillary area, which is available for fluid exchange (47). The hydrodynamic conductivity or the filtration properties of the capillary walls seem to remain unaffected by a lowered temperature (48,49).

ACID-BASE STATUS AND BLOOD GAS TRANSPORT

The relation between plasma pH, plasma partial carbon dioxide tension (P_{CO2}) and the plasma HCO_3^- concentration is given by the

equation of Henderson and Hasselbalch :

$$pH = pK'_1 + \log c^p_{HCO_3^-} / s'.P_{CO_2}$$

where pK'_1 is the -log of the apparent over all first distribution constant of CO_2, $c^p_{HCO_3^-}$ is the plasma bicarbonate concentration (mmol/l) and S' the solubility of carbondioxide in plasma (mmol/l.kPa). The values of pK'_1 and S' are temperature dependent.

It has been shown that pH rises when temperature falls. The rate at which pH increases with decreasing temperature ($\Delta pH/\Delta T$) depends upon the initial temperature and upon the effects of temperature on the CO_2/HCO_3^- equilibrium and the haemoglobin and protein buffer systems: When hypothermia becomes deeper, the ionization of haemoglobin and other proteins is reduced, whereas more bicarbonate ions are formed and the carbondioxide-carrying power of the blood increases (50). Several investigators studied the temperature dependence of the pH and found a $\Delta pH/\Delta T$ of approximately -0.014 to -0.015 pH unit per degree centigrade in blood (51,52,53,54,55,56). The $\Delta pH/\Delta T$ decreases when the concentrations of haemoglobin and other proteins decrease. For plasma lacking protein, with an initial pH (at 37°C) of 7.4, $\Delta pH/\Delta T$ is about 0.012 to 0.014 (54,55). For simplified interstitial fluid, containing little or no protein, the $\Delta pH/\Delta T$ is about -0.012 (51).As to the control of the acid-base status, it is generally agreed that the "normal" pH at 37°C is about 7.4. However, when the temperature of the whole body or the isolated organ, as in organ preservation, falls, the optimal value of the pH is less obvious (6,57,58,59,60). In this

connection, it is an interesting suggestion that the proper reference value may be the electrochemical neutrality, i.e. where pH = pOH (61) and that, as in normothermia, the pH of blood or plasma remains 0.6 pH units alkaline to the neutral point at all temperatures. Upon cooling the pH of the neutral point rises. This is paralleled by an increasing pH of blood or plasma (Fig. 1-2). In organ preservation, this idea gains importance in view of the consideration that an isolated organ behaves like a poikilotherm, and that the blood of poikilotherms becomes more alkaline in hypothermia. This suggests that the optimal pH for hypothermic preservation, in particular during hypothermic perfusion, is in the alkaline range (57,62).

When hypothermia deepens, the oxygen dissociation curve shifts to the left, indicating that at lower partial oxygen tensions oxygen will not readily be released by haemoglobin to the tissues. The oxygen availability appears, however, to be sufficient, facing the drastically reduced needs of tissues for oxygen (9,63), and the increased solubility of oxygen in plasma in hypothermia (64).

PRESERVATION MEDIA

NATURAL MEDIA

Blood

Theoretically, blood should be the preservation medium of choice, particularly in normothermic perfusion preservation.It provides for instance, in normothermia an optimal buffering and oxygen-carrying capacity by its content of proteins (haemoglobin). However, in hypothermia the need for oxygen carriers decreases

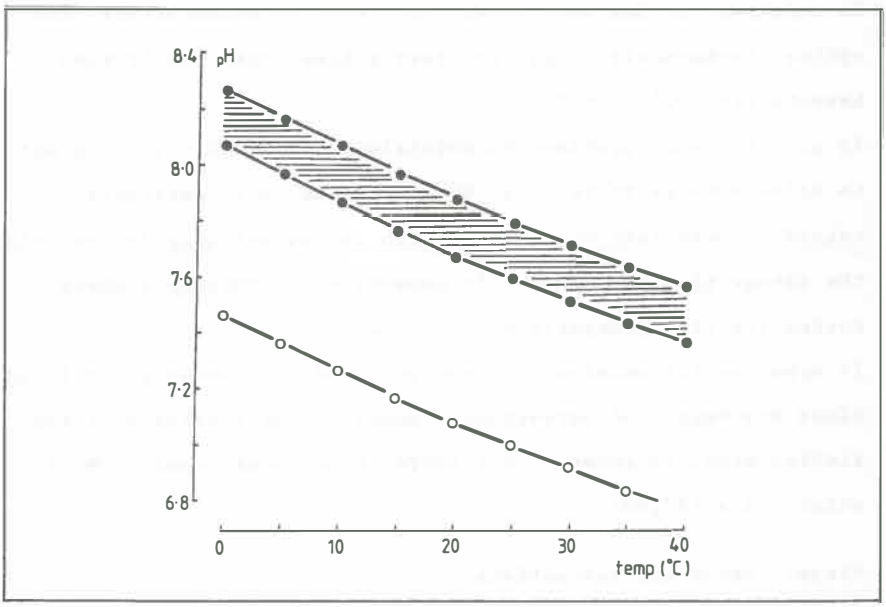


Fig.1-2: The relation between body temperature and neutral pH (after 104;○) and blood (after 101;●): pH of blood is always 0.6 pH units alkaline to pH of the neutral point. The hatched area indicates the normal range of the blood's pH.

because the metabolism slows down and the quantity of freely dissolved oxygen in plasma increases. In addition, the carbon dioxide transfer diminishes in hypothermia; the oxygen dissociation curve shifts to the left such that, at any reasonable pH, little oxygen is transferred to the tissues, and hardly any carbondioxide is carried away. Therefore, haemoglobin also looses its buffering capacity in hypothermia (65). Furthermore, the presence of blood corpuscules may cause, particularly in hypothermia, unphysiologically high pressures and intravasal sludging by increasing the viscosity of the medium. This may produce deleterious effects to the perfused organ (66).

In addition, it has been shown that, even in normothermia, the optimal haematocrit (0.20) is clearly lower than the "normal" haematocrit (0.40) (67).
In normothermia, problems in maintaining organ vitality are apt to arise because it is still impossible to build artificial reconditioners that can compete with the animal body in restoring the damage that is produced by pumping blood through systems, containing bio-incompatible materials.
In experimental studies on liver preservation, whole and diluted blood has been used extensively. However, the results of these studies were, in general, not better than those obtained with other media (57,68).

Plasma, serum and derivatives

Plasma, platelate rich or lacking platelets, has drawn much attention in clinical as well as in experimental preservation (57,69,70).
The rise in vascular resistance, as seen in perfusion with plasma and blood, has been attributed to the presence of particulate matter, that could be visualized in fat stained sections of tissue. It has been concluded that deposition of unstable lipoproteins was the major cause of the rising perfusion pressure, particularly in hypothermic perfusions (6). These lipoproteins could be removed by cryoprecipitation or by treating the plasma with silica gel (71,72).
Although cryoprecipitated plasma has shown good results in experimental and clinical preservation, there are also some clear disadvantages linked to its use, including the time consuming

preparation and the imperfection of the precipitation technique (6). Further disadvantages are, as with all other natural media, the high titers of blood group agglutinigens and the always existing risk of transferring pathogenic germs (71).

Many organs have been preserved with silica gel treated plasma (72,73). Good results were obtained especially when media were hyperosmolal and contained high concentrations of potassium. The conclusion that silica gel treated plasma is superior to other media is, however, disputable.

The most natural way to remove fibrinogen from plasma is converting it to serum. Although successful longterm perfusions with serum have been reported (59), it is hardly used as a preservation medium because it bears most of the disadvantages that are well known for natural media. In contrast, plasma protein fraction (PPF), a serum like solution, prepared in a two step procedure of ethanol extraction and pasteurization -i.e. incubation at 60°C- of plasma, has been used extensively in organ preservation. Some investigators found PPF to be inferior to cryoprecipitated plasma because of the presence of antibodies against the perfused organs (57) . On the other hand PPF was reported to be superior to cryoprecipitated plasma because proteolytic enzymes, which may damage cell membranes, are absent (74). Furthermore, PPF showed a vasodilator effect, due to the presence of bradykinine (75,76) when it was used as an infusion solution. It is, however, questionable if PPF produces this effect also in hypothermia.

Further simplification of preservation fluids led to media that contained albumin as colloid only (77). Excellent results were

obtained with media containing 45 grams albumin per liter. The specific action of albumin in these simple solutions was thought to be twofold: the provision of oncotic pressure and the stabilization of membranes (71). In addition, albumin might act as carrier protein, e.g. of pharmaca.
Albumin containing solutions are prepared easily, can be shelf stored for a relatively long period of time, are constant in composition and are free of hepatitis virus and cytotoxic antibodies. There are, however, also several disadvantages. It has been demonstrated that during perfusion albumin escapes from the intra- to the extravascular space, thus reducing oncotic pressure (74). This effect is enhanced by perfusion induced denaturation of albumin. The products of denaturation may even cause endothelial damage (78). At present there is, however, no acceptable substitute for albumin (79).

ARTIFICIAL MEDIA

Electrolyte solutions; extracellular type

The extracellular type of electrolyte solutions are only of interest as far as it concerns mechanical perfusion. The outcome of perfusions with these media has been shown to depend on the type of organ perfused. Several organs, e.g. the liver, appeared to do better when they were perfused with richer media, i.e. media containing oncotically active and/or metabolism supporting substances (71,80).

Electrolyte solutions; intracellular type

The intracellular type electrolyte solutions were developed for use in simple cold storage of organs. In perfusion preservation these solutions have not been shown to be superior to the extracellular type solutions (71).

Three currently important theories to explain the action of these solutions concern the prevention of cellular swelling, the conservation of cellular energy resources and the avoidance of noxious changes in the intracellular ion content respectively. Probably the major benificial action of these media is the prevention of cellular swelling in hypothermia (57).

The optimal concentrations of ions to mimic the intracellular fluid are not fully known. Most solutions contain high concentrations of potassium and low concentrations of sodium, and are moderately to strongly hyperosmolar. Discussions continue wether magnesium, phosphate, sulphate and citrate should be added (57,69,81,82,83,84,85).

There is some agreement that hyperosmolar solutions, containing high concentrations of potassium may be used in simple cold storage as well as in perfusion preservation (86,87,88), although they do not offer specific advantages over the extracellular type solutions. A prerequisite for both types of solutions in perfusion preservation has appeared to be that they contain an oncotically active substance (89).

As the intracellular type solutions were primarily developed for the use in hypothermia, it might be expected that they are of lesser use at higher temperatures. Recently this hypothesis was substantiated (90).

Tissue culture media

Tissue culture media present an intermediate between the very "meager" electrolyte solutions and the presumably nutrient rich natural media. They contain numerous substances that have been identified as be essential for cellular functioning and growth. In organ preservation, these media have been used seldomly. Long before the era of organ transplantation successful perfusion experiments with tissue culture media derived perfusates were done (91). Interesting results were reported on the preservation of intestinal segments, livers and lymfoid tissue (92,93). Nevertheless, there is a rational for the use of perfusates, rich in nutrients, in particular when one considers preservation temperatures above 10°C.

Since it has been noticed that in deep hypothermia metabolism still continues (63), and because metabolic activity increases when temperature rises, the maximum preservation time will be limited by the perfusate's content of nutrients. Hence, inclusion of nutrients in the perfusate will appear to be crucial when aiming at long term preservation, i.e. preservation periods beyond twenty hours when it concerns livers.

Little work has been done on the need for nutrients in hypothermia. Kidneys prefer short chain fatty acids and ketone bodies -i.e. caprylate and beta-hydroxybutyrate- in hypothermia (26). Nevertheless, they are, depending on the relative concentrations of the different substrates, capable of utilizing glucose and longer chain fatty acids as well under these circumstances (94). However, unphysiologically high concentrations of fatty acids are needed before extraction from

the perfusate can be demonstrated (31).
In livers, fatty acid combustion seems to be preferred over carbohydrate oxidation in hypothermia (95).
Little work has been done on protein and amino acid metabolism in hypothermia. There are indications that a negative nitrogen balance in hypothermia can be corrected by supplementation of amino acids (96); excellent results with an amino acid containing medium were reported in heart preservation (97).

ADDITIVES TO PRESERVATION MEDIA

To improve the quality of preservation media, many substances have been added to these fluids on the basis of their pharmacological, biochemical or physiological actions. Referring to the intention with which these substances were added, four main groups may be distinguished: Control of (micro-) circulatory obstruction, cell protection, metabolic support, and metabolic inhibition. A heterogeneous fifth group consists of substances that do not fit in one of the foregoing groups.

Control of (mikro-) circulatory obstruction

Anticoagulants

Heparin sodium has been widely used, although its usefulness is disputable. When the pH falls below 6.5 heparin is inactive (98,99). Furthermore, once fibrin is formed, heparin is useless. Therefore fibrinolytic activators have been tested with favourable results (90).
Platelet aggregation has been successfully inhibited with dipyridamole (69) and prostacyclin (PGX) (99,100) the most potent

platelet aggregation inhibitor discovered so far (99). The applicability of PGX, however, is restricted a little by its biological instability (101). Studies on more stable PGX analogues have shown promising results (102). In addition, PGX also acts as a vasodilator (101).

Vasoactive drugs

Alpha and beta blocking pharmaca have been used to prevent and to reverse vasoconstriction. Which of the two types of blockers will be effective depends on the type of receptor, found in a particular organ. Because beta receptors predominate in the liver, norepinephrin causes vasodilation in this organ (69). Propranolol, a beta blocking agent, contrarily, causes vasoconstriction (57,68,69,103). The action of alpha receptor blocking agents in liver preservation is not evident and not clearly understood.

Synthetic local anesthetics are known to cause vasodilation. Therefore, procaine has been added to wash out solutions for kidney preservation (104). Only high dosages of procaine -i.e. 10%- produced any effect. However, high dosages of procaine may be noxious (57,69).

Prevention of vascular occlusion may be part of the beneficial effect of calcium overload blockers. Intracellular calcium overload has been shown to play a crucial role in the cytotoxic mechanism, that leads to anoxic cell death (105). Pharmaca have been developed, that prevent the occurrence of an excessive calcium influx by blocking calcium channels, or that prevent the the formation of the calcium-calmodulin complex by inhibiting

calmodulin (106). In organ preservation, however, experience with these drugs is too meager to warrant definite conclusions. Nevertheless, interesting results have been reported from the use of the calmodulin inhibitor trifluoperazine (TFP) (107), although it may well be that its primary action is membrane stabilization (80).

Cell protection

The integrity of cells can be protected by agents that "stabilize" membranes, that prevent the formation of oedema, and that protect against direct toxic effects of certain substances.

Membrane stabilization

The membrane stabilizing effect of corticoids in organ preservation is not clear, since there are indications that they are only protective at low dosages, cause lysosomal disruption at high dosages and are capable of increasing vascular resistance during perfusion (57,69,109).

It has been reported that membrane stabilization could be achieved by replacing the water of preservation media by deuterium oxide (D_2O) (110). D_2O replaces H- by D-linkages in biological makromolecules, thus stabilizing their structure. In the preservation of livers, kidneys and hearts D_2O has shown promising results (86,110,111); cellular oedema was reduced and the adenine nucleotide content of the D_2O treated organs was higher.

Prevention of oedema

Inclusion of oncotically active substances in preservation media is the oldest way to reduce cellular oedema. In this approach, the carbohydrates -i.e. glucose, sucrose, mannitol and dextrans- present an important group. Hypertonic mannitol solutions have been shown to be effective, even in reducing the thickness of normal endothelial cells, thus reducing vascular resistance below normal. Therefore these solutions may play a role in preventing the so called "no reflow phenomenon" (57,69,79).
Recently the group of Belzer reported extremely good results with their "UW-solution", which contains high energy metabolic intermediates and lactobionate and raffinose as new impermeants (158,158A,158B). It is probably the action of the impermeants which makes this solution superior to all the other preservation fluids, that have been developed so far (158C). Other oncotically active substances such as albumin have been discussed before.

Cytoprotection

It has been recognized that non-flow hypoxia may create a condition within the cell in which molecular oxygen is reduced to toxic free oxygen radicals, resulting in peroxidative injury to biomembranes and alterations in permeability (112).
To prevent the generation of oxygen free radical species, favourable results have been seen from the administration of Coenzyme Q, one hour before the induction of ischaemia. Coenzyme Q protects mitochondrial function, improves energy metabolism and reduces peroxidation during reperfusion following non flow ischaemia (113).

Attempts to remove oxygen derived free radicals by free radical scavengers have shown promising results. Catalase (CAT) and superoxide dismutase (SOD) limited lung injury for up to five hours of non flow ischaemia when added before the start of reperfusion (114,115). SOD has been shown to improve organ preservation in liver transplantation. In this respect, also the favourable results of acceptor treatment with allopurinol, a known metabolic inhibitor of xanthine oxidase, have been reported (116). Vitamin E also prevented damage by scavenging free radicals, but has the practical disadvantage that it has to be administered several days before the induction of ischaemia (115).

Metabolic support

Hormones

Most experience has been gained with the inclusion of insulin in preservation media (6,57,69,92,117). The metabolic activity of insulin is negligible below 25°C (118,119).

Oxygen carriers

To substitute for the function of hemoglobin in the transport of oxygen and carbon dioxide, fluorocarbon emulsions have been studied experimentally for many years (120,121,122,123,124). They have shown an excellent oxygen carrying capacity, thus stimulating metabolism, in particular in normothermia (125,126). Recently, the Cambridge group reported successful 48 hour preservation of rat liver by perfusion with a perfusate containing FC-43, a fluorocarbon emulsion (127). No adverse

effects have been observed on cellular integrity and organ function (128), although a reduction of the capacity of the reticulo endothelial system has been reported (129). However, it is still unclear if fluorocarbon containing media are entirely non toxic (130).

Intermediary metabolites

To maintain or even increase tissue levels of ATP, intermediary metabolites have been included in preservation media. Oxaloacetic acid, alpha oxoglutarate, and citrate served this purpose well (57), but have been superseded by substances that are more directly involved in the production of high energy metabolic intermediates.

High energy metabolic intermediates

Principally there are three ways to support high energy metabolism: The administration of adenine nucleotides (ATP,ADP,AMP), the inclusion of precursors (adenosine, phosphate, inosine, hypoxanthine) in the perfusate, or blocking the final degradation of xanthine to uric acid.

The administration of ATP has been useful when ischaemia played a prominent role (57,24,131). ATP was superior to ADP and AMP (117).

Addition of adenosine appeared only to be benificial when it was combined with phosphate (132).

Although the benificial effect of inosine was attributed to improvement of the regeneration of adenine nucleotides during ischaemia (134), recent studies indicate that inosine accelerates adenine nucleotide resynthesis after recirculation by inducing

vasodilation (222).

The simplest way to prevent the loss of adenine nucleotide precursors is to block their degradation to uric acid. For this purpose allopurinol has been administered before the onset of ischaemia and/or included in the preservation fluid (109,133). The action of allopurinol can be explained by the inhibition of the enzymes 5'-nucleotidase and xanthine oxidase (135). In addition, xanthine oxidase is known as a biological source for superoxide *in vitro* (124,109). Therefore, it is tempting to conclude that allopurinol also plays a role in the scavenging of oxygen radicals.

Metabolic inhibition

Metabolic inhibitors have been used to reduce cellular metabolic rates in normothermia to levels comparable with metabolic rates in deep hypothermia. Many agents have been used for this reason, e.g. magnesium, fluoride, potassium chloride and phenothiazines (57,69). The latter is probably most effective in the intact animal body.

Miscellaneous

Antimicrobial and antimycotic drugs have been added to preservation media to prevent bacterial and mycotic overgrowth. Several combinations of drugs have been used (57,69). The action of antibiotics is affected below 25°C (136) because at this point a significant reduction of bacterial growth and toxin production occurs.

To provide a visual aid for pH control some investigators have

included a pH indicator in the preservation fluid (6). However, substances as phenosulfophtalein are excreted into the bile. This process is partly energy dependent. Therefore, phenosulfophtalein containing pH indicators may be less suitable in liver preservation.

PRESERVATION METHODS

SIMPLE COLD STORAGE

In simple cold storage organs are, after being washed out and initially cooled, stored on ice in an intracellular type of electrolyte solution, without further perfusion. This method derives its efficacy primarily from a reduction of metabolic activity by cooling, although some metabolism still exists in profound hypothermia (63). In this way organs are protected against the injurious effects of anoxia.

In general, the degree of protection afforded by simply cooling parallels the level of hypothermia to which an organ is cooled (57). Therefore, the lowest temperature above freezing point has, logically, been assumed to be the optimum temperature for simple cold storage. With cooled washout solutions, about two minutes are required to let the core temperature of kidneys drop below 10°C. By additional surface cooling, storage temperatures can easily be maintained in the range of 0 to 6°C for several hours (57). It may be anticipated that for livers the situation is by and large the same.

The advantages of simple cold storage are its simplicity, its low

costs and the ease of transporting the organ.
Disadvantages are the continuing metabolism in profound hypothermia which presents a finite limit on the duration of organ storage by this preservation technique, and the limited applicability in the preservation of organs that were subjected to prolonged periods of normothermic non flow anoxia (137).

PERFUSION PRESERVATION

Intermittent perfusion

Intermittent perfusion stands between simple cold storage and continuous perfusion, because the organ is perfused only during a small fraction of the entire preservation period. The introduction of this technique was based on two assumptions: the removal of waste products of metabolism combined with the supply of fresh nutrients, and the limitation of vascular damage, thought to be caused by continuous mechanical perfusion (68,138). With the so called "squirt" perfusion (138), in which an initial cooling flush is followed every five minutes by a 15 second "squirt" of 20 ml perfusate, only few investigators have obtained promising results in liver preservation (139,140,141).

Continuous perfusion

Ex vivo perfusion

In ex vivo perfusion an organ is connected to the circulation of a perfusor animal through an arterio-venous shunt. With this technique hearts, kidneys, livers and pancreases have been maintained vital for hours to several days (142,143,144,145,146,147). The most important applications of the

ex vivo perfusion lies in the support of the animal body when organ function failed (142,143,144) and in the study of organ function in normothermia (148).
In the preservation of human cadaveric organs ex vivo perfusion is unlikely to be practical because a xenogeneic perfusor animal would be needed (57), presenting major problems of cross-species immune responses.
Recently, a combination of continuous hypothermic perfusion and normothermic ex vivo perfusion, called intermediate ex vivo perfusion, was introduced in heart and kidney preservation (97,149). Attemps to make this method applicable in clinical preservation by replacing the perfusor animal by a heart lung machine -i.e. intermediate in vitro perfusion- were less promising (149).

Intermediate host preservation

In intermediate host preservation, also called in vivo storage or "live" preservation, organs are stored by temporary transplantation into an intermediate host. Theoretically, this method might have some advantages. However, the duration of in vivo storage is limited by the onset of rejection.

Continuous mechanical perfusion

In continuous mechanical perfusion preservation the isolated organ is connected to a machine for extracorporeal circulation, which basically resembles the conventional heart-lung machine. Mostly the perfusion fluid is recirculated.
The construction of the first entirely adequate perfusion and oxygenation system may be credited to Lindbergh and Carrel. They

built a system whereby perfusion fluid was well oxygenated and forced by compressed gas (91). The first successful preservation of organ, i.e. kidneys, by continuous mechanical perfusion was achieved by Humphries not until 1964 (150).Since then, many investigators have studied the applicability of continuous mechanical perfusion in organ preservation. From their experiences several criteria for successful perfusion preservation emerged. Some of them are discussed in the following paragraphs.

CIRCUITRY

Perfusion circuitry is mostly made of synthetic materials which may cause problems of toxicity, permeability to gasses and absorption of perfusate constituents.

Silicone rubber has been widely employed, because it has been proven not to be toxic and because of its general acceptance for surgical purposes. Nevertheless, this material is not ideal because of its extremely high permeability to oxygen and carbon dioxide, as illustrated by the use of silicone rubber in membrane oxygenators (151).

Polyvinylchloride (PVC) is also permeable to oxygen and carbon dioxide. Furthermore, it has the disadvantage of containing plasticizers, stabilizers and lubricants, which may be toxic (152). Another disadvantage, which PVC shares with silicone rubber, is the high affinity for fatty acids (153,154). This makes these materials less suitable for hypothermic perfusion, when tissues generally prefer fat combustion over glucose

oxidation.
Polyethylene, not possessing the disadvantage of fat adsorption, is less suitable for organ preservation because of its high permeability to gasses and its low melting point, which makes heat sterilization difficult (154).
All these problems are not encountered when nylon is used.

PERFUSION DYNAMICS

It is questionable whether low flow perfusion should be preferred over high(er) flow perfusion, and whether pulsatile or non-pulsatile perfusion should be employed. On the assumption that high flow rates may cause vascular injury, several investigators employed trickle and continuous low-flow perfusion (120,139). However, at subnormal flow rates oxygen delivery may become insufficient, which may be prevented by inclusion of oxygen carriers in the perfusate (120).
Pulsatile perfusion has been considered for a long time to be essential for normo- as well as hypothermic perfusion (6). There are several reports that indicate that non-pulsatile perfusion is equivalent to pulsatile perfusion (57,59,155), and that pulsatile perfusion is not essential for long term perfusion preservation (57).

CONDITIONING OF THE PERFUSATE

A constant composition of the perfusate is an essential prerequisite for successful preservation. The easiest way to guarantee constant levels of the several constituents and the

removal of the waste products of metabolism is changing the perfusate regularly or continuously. Single pass and intermittent perfusion, and the periodic exchange of pefusates have all been tried (60,68), but bear the obvious risk of washing out valuable metabolites, such as hypoxanthine. A possible answer to this is the inclusion of a dialyzing unit in the circuit. In isolated rat liver perfusion, this has shown promising results (101).

PRESERVATION OF THE LIVER

Liver preservation for clinical transplantation is currently performed by simple cold storage with an intracellular type electrolyte solution or with plasma protein fraction.
In essence, the basic principles for liver preservation are the same as those for the preservation of other organs. Special requirements, however, will be needed for the positioning of the liver during long term perfusion.

SIMPLE COLD STORAGE

Because of its size, the liver can only be cooled by immersion in the preservation fluid. Simple extracellular type solutions enabled to extend the period of tolerance to ischaemia to about two hours (1,57). With a technique in which the liver was washed out through the portal vein with a dextran containing extracellular type electrolyte solution, followed by a plasma-bicarbonate-glucose solution livers have been preserved moderately successful up to eight hours (2,156,157). The best results have been obtained when the potassium concentration and the osmolality of the solutions were increased (57). In this

respect, the group of Belzer reported extended preservation of the liver by storage in a new preservation medium ("UW-solution"), that contains lactobionate and raffinose as impermeants to prevent hypothermia induced cellular swelling (158).

PERFUSION OF THE LIVER

Experimental hypothermic continuous mechanical perfusion with natural perfusates produced preservation periods up to 48 hours (68,127). When employing artificial perfusates results have been inconsistent up to ten hours of preservation and poor when the preservation period approached 24 hours (68). Intermittent perfusion has not improved these results (139,140,141). Nevertheless, there are, when conservation of cellular ATP is taken into account, strong indications that continuous perfusion is a feasible method for long term preservation (159).

POSITIONING OF THE LIVER

Disturbance of the microcirculation, resulting from inadequate positioning of the liver (160) may be one of the causes of the poor results of long term perfusion preservation. Several investigators have attempted to improve the suspension of the liver during preservation. It has been tried to spread the pressure by softening the liver support. Placing the liver on a dish like support (142,161), a plastic membrane that was spread in a metal frame (162), a rubber screen (163), or a perforated support (164,165) have, however, proved to be of little value in this respect.

More promising results have been achieved when anatomical, morphological and haemodynamical features of the liver were taken into account. To mimic the normal situation in-vivo, several workers have developed "liver chambers", and preservation methods, in which the liver was suspended softly in a humid environment (144,166,167,168), and in which the liver was continuously subjected to intermittent external pressure changes (165,169,170,171,172).

Although the liver's microcirculation has been improved by these methods, entirely satisfactory results have not yet been obtained.

SECTION II

OUTLINE OF THE MODEL

CHAPTER 2

THE MODEL OF ISOLATED RAT LIVER PERFUSION IN NORMO- AND HYPOTHERMIA

INTRODUCTION

Although isolated liver perfusion is performed since halfway the last century (5,173), isolated rat liver perfusion is an attainment of the last five decades. The first apparatus for rat liver perfusion was constructed in the 1940s (174). However, isolated rat liver perfusion did not become popular as a research tool until the early fifties, and since, many apparatuses have been developed for physiological, biochemical and pharmacological purposes (5).

After experimental and clinical liver transplantation programs started, isolated rat liver perfusion became also an useful technique for studies, that aim at extension of the preservation time of livers for transplantation (e.g. 120). Because hypothermia plays a keyrole in liver preservation, it is obvious that for the application of isolated rat liver perfusion as a research technique in this field, apparatuses have to be designed that meet the requirements of (rapid) cooling and controlling the subnormal temperatures between narrow limits.

This chapter presents the method of isolated rat liver perfusion and hepatectomy as used in the experimental studies, described in the following chapters.

THE APPARATUS

The core of the apparatus (Fig. 2-1), in which two livers can be perfused simultaneously in an unidirectional manner, is formed by two symmetrically placed, transparant organ chambers (Fig. 2-1g), provided with a support to put the liver on (Fig. 2-1h). The

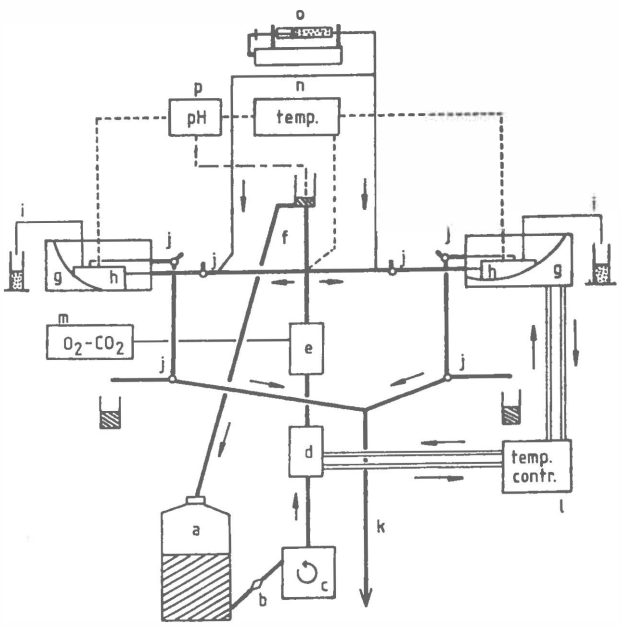


Fig. 2-1: The perfusion apparatus. The set up, presented here is suitable for double single pass perfusion.
a. reservoir; b. filter; c. roller pump; d. heatexchanger; e. membrane oxygenator; f. overflow system; g. organ chamber; h. liver support; i. bile cannula; j. three way stopcocks; k. drain for the outflowing perfusate; l. temperature control unit; m. oxygen and carbon dioxide cylinders; n. electric themometer; o. infusion pump; p. potentiometer.

have a coil of tubing through which cooled or warmed water circulates to control the ambient temperature of the perfused liver. This makes it unnecessary to put the apparatus in a thermostatically controlled cabinet. Thanks to the use of separated heating and cooling devices, it takes little time to bridge large temperature intervals, e.g. 30 °C within 20 minutes. The perfusate is pumped by a roller pump (Fig. 2-1c) from a large reservoir (Fig. 2-1a), which is placed on an electromagnetic stirrer to guarantee homogeneous mixing. The fluid is, subsequently, forced through a heat exchanger (Fig. 2-1d) and a membrane oxygenator (Fig. 2-1e).

Behind the oxygenator an overflow (Fig. 2-1f) is incorporated in the circuit. Thus, it is possible to vary the hydrostatic pressure with which the liver is perfused. The overflow system also enables rapid (re-)circulation of the perfusate, thus achieving efficient mixing of added substances. Furthermore, the overflow acts as a simple bubble trap and pulse damper.

After passage through the liver, the perfusate is, in the single pass set up, drained off. It is also possible to lead the perfusate back to the reservoir, thus making the apparatus a single recirculating system. To remove particles from the perfusate, a disposable filter of the type used in infusion systems is placed between the reservoir and the roller pump (Fig2-1b). For sampling and direct flow measurements, threeway stopcocks are incorporated in the circuit before and behind the organ chambers (Fig. 2-1j). The bile, produced by the perfused liver, is drained off by a separate cannula (Fig. 2-1i).

The circuitry consists of nylon and silicon tubing. Behind the

oxygenator all tubing is nylon, to minimize diffusion of gasses (chapter 1).

To monitor the pH of the perfusate, and to monitor the temperature of the perfusate and the liver, a H^+-electrode (Fig2-1p) and an electric thermometer (Fig 2-1n), provided with three thermistor probes, are added to the apparatus.

For the administration of bile acids and other substances, an infusion pump (Fig.2-1o) is connected to the perfusion system immmediately before the organ chambers.

THE PERFUSION MEDIUM

Eagle's basal medium (Gibco, Glasgow; table 2-1) in Earle's salt solution is used. This is a type of perfusate intermediate between blood and the simple electrolyte solutions (chapter 1). To the medium extra glucose (up to 10 mM), insulin (2 IU/l), albumin (2g/l), ampicillin (10 ug/l) and sodium bicarbonate (a minimum of 17 meq/l) are added.

Table 2-1: Composition of the perfusate; Eagles basal medium.

Ingredient	mg/l	Ingredient	mg/l
Arginine HCl	21.06	Biotin	1.00
L-Cystine disodium salt	14.21	D-Ca pantothenate	1.00
L-Glutamine	292.30	Choline chloride	1.00
L-Histidine HCl.H2O	10.50	Folic acid	1.00
L-Isoleucine	26.23	i-Inositol	1.00
L-Leucine	26.23	Nicotinamide	1.00
L-Lysine HCl	36.53	Pyridoxal HCl	1.00
L-Methionine	7.46	Riboflavin	0.10
L-Phenylalanine	16.51	Thiamine HCl	1.00
L-Threonine	23.82		
L-Tryptophan	4.08		
L-Tyrosine	18.11		
L-Valine	23.43		

The osmolality of the perfusate is about 300 mOsm/kg.
The perfusate contains a little albumin, since complete omission may disturb synthetic pathways, e.g. the synthesis of fatty acids (175).

CONTROL OF THE PERFUSION CONDITIONS

The temperature control system of the apparatus presents a unique feature as compared to that of conventional apparatuses for isolated rat liver perfusion. The installation of a heat exchanger in the circuitry and of a heating/cooling coil in the organ chambers, enables efficient control of the temperature of the perfused liver by internal and external cooling or heating; deviations in temperature are adjusted manually by changing the temperature of the water that flows through the heat exchanger and the heating/cooling coils.
The hydrostatic pressure under which the perfusate flows through the portal vein is fixed at about 1.3 kPa (13 cm H_2O). The perfusion characteristics of the cannula, fixed in the portal vein, are such that there is a linear relation between pressure and flow in the physiological pressure range (Fig.2-2).
Constancy of the perfusate pH is of great importance for liver cell metabolism (176). In this model pH is fixed at 7.4 as measured at 37°C. At lower temperatures the actual pH of the perfusate is adjusted such that it remains about 0.6 alkaline to the neutral pH at that point (61) (chapter 1). Because the perfusate contains little protein, the pH increase with cooling is less prominent than in blood (51). This is taken into account

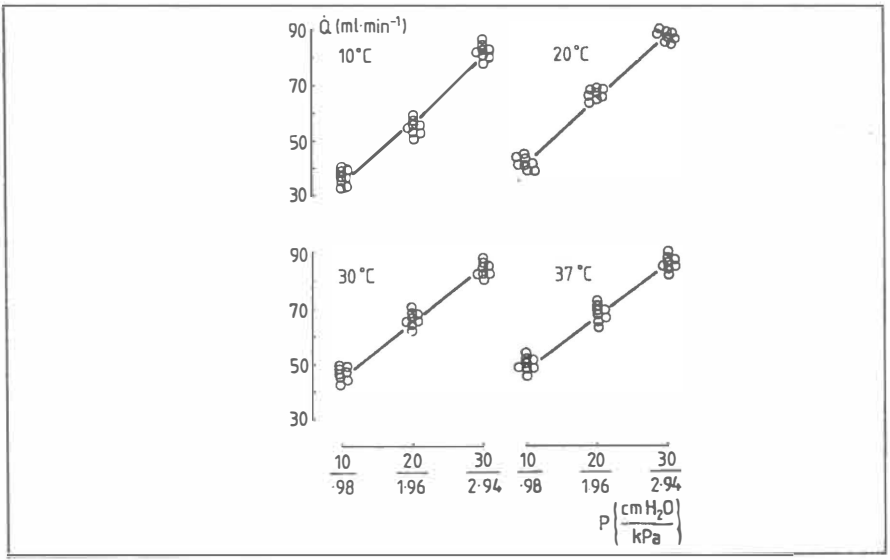


Fig. 2-2: Perfusion characteristics - i.e. the relation between the perfusion pressure (cm H20/kPa) and the perfusate flow (Qp)- of the cannula, fixed in the portal vein at 10,20,30 and 37°C. n was always 8.

when adjusting the perfusate's pH (Fig. 2-3).

The pH is adjusted by changing the ratio carbon dioxide to oxygen in the gasmixture with which the perfusate is equilibrated. In the single pass set up the bicarbonate concentration is assumed to remain constant.

Because the perfusate is free of oxygen carriers, the partial oxygen tension (P_{O2}) is kept high. The oxygenator, used in the present set up, has such a capacity that high oxygen tensions can be reached; about 70 kPa at 37°C. P_{O2} increases as temperature decreases. No attempts are made to reduce high P_{O2}. The combination of a high P_{O2} and, because of the low viscosity of the perfusate, high perfusate flow guarantees adequate

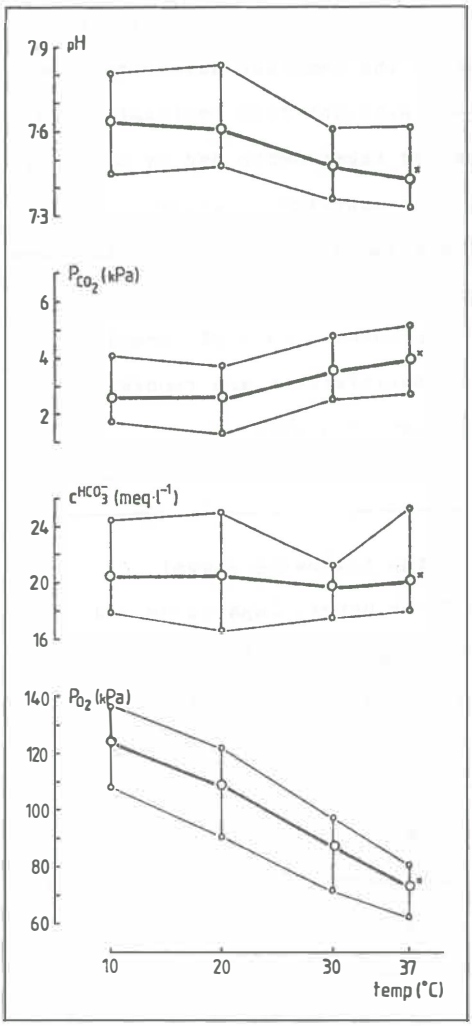


Fig. 2-3: Median pH, carbon dioxide tension (PCO2), oxygen tension (PO2), and bicarbonate concentration (cHCO3) of the inflowing perfusate at 10, 20, 30 and 37°C. The number of measurements was 40, unless indicated otherwise. * n=48.The vertical bars indicate the range.

oxygenation of the liver tissue. Furthermore, it has been shown that high concentrations of oxygen may prevent ischaemic damage as indicated by free fatty acid accumulation in the perfusate (177).

Data on oxygenation and acid-base status of the inflowing

perfusate in normo- and hypothermia are presented in Fig. 2-3. Microbial growth, in particular in the membrane oxygenator, may present problems, especially when nutrient rich perfusates are used. By working cleanly during the experiments and by cleaning the apparatus thoroughly after each experiment, gross contamination of the system is prevented. The policy is to rinse the apparatus with tap water immediately following each experiment. Then, the system is perfused with a 8% formol solution. Finally, traces of the desinfectant are removed by rinsing the system with sterile distilled water.

HEPATECTOMY AND CANNULATION

The surgical procedure comprises the following steps: anesthesia, preparation of the liver donor, laparatomy and mobilization of the liver, cannulation of the bile duct, the portal and the thoracïc inferior caval vein, excision of the liver, and, finally, the transfer of the liver to the organ chamber.

Anesthesia is induced and maintained with ether. Firstly, the rat is put in a pot, containing cotton gauze, saturated with ether. Then, anesthesia is continued by placing the rat's nose in a small cylinder, packed with ether soaked gauze.

The efficacy of the anesthesia is reflected in the loss of the lid reflex, the absence of reaction to painbringing stimuli -e.g. pinching the tail- and the complete relaxation of the cervical muscle. When anesthesia is too deep, respiration becomes irregular and difficult, while the color of the blood of the abdominal organs darkens. At the other hand, the frequency of

respiration and the heart rate increase, and the rat may even show reflectory movements when anesthesia is too shallow and the rat senses pain.

After the rat is weighed, the abdominal and thoracic walls are shaved and desinfected by swabbing with 96% ethanol. To achieve an optimal presentation of the liver during operation, the rat is placed in supine position on a roll of surgical gauze at the lumbar region.

Once anesthesia is adequate, the abdomen is opend by a bilateral subcostal incision. The abdominal wall is retracted laterally and caudally. The liver and surrounding tissues will protrude from the abdomen by the elevation of this region due to the roll of gauze on which the rat is placed.

Then, the liver is mobilized by freeing it from its ligaments and surrounding structures. The falciform ligament is divided as far as the caval vein. The ligament, attaching the left lateral lobe of the liver and the posterior wall is cut. The stomach is pulled forward to allow the division of the pars flaccida of the lesser omentum and of the fibrous ligament, which joins the anterior part of the caudate lobe and the stomach. After the stomach and the intestines are retracted to the left, the portal region, comprising the portal vein, the hepatic artery and the common bile duct, becomes visible.

Bile duct cannulation is then performed. Two ligatures are placed around the common bile duct. The most distal one is tied, whereafter the bile duct expands. Then, choledochostomy is done with a pair of fine iridectomy scissors. This is followed by cannulation with a flexible cannula (Silastic, Dow Corning Corp.;

OD 0.9 mm, ID 0.5 mm), provided with a hard polyethylene tip (Portex Ltd.; OD 1.0 mm, ID 0.5 mm). The tip of the cannula is advanced up to about 2 mm proximal to the bifurcation of the bile duct, and secured by ligating the tie. The distal ligature is tied as a stay ligature. When the cannula is in place, bile will drip immediately.

Then, loose ligatures are placed around the portal vein (PV), the superior mesenteric vein (SMV), and the abdominal inferior caval vein (VCIA). The ligature around the PV also includes the hepatic artery (HA), which runs immediately posterior to the PV. The ligature around the VCIA runs just superior to the entrance of the right renal vein into the VCIA.

For cannulation of the PV, the ligature around the SMV is tied firstly. By this, the liver is not disconnected from blood supply, since the splenic vein and the HA remain patent. Just proximal to the SMV ligature, the anterior wall of the PV is picked up with a forceps and cut with iridectomy scissors. The dripping polyethylene cannula (Portex Ltd.; OD 2 mm, ID 1.5 mm) is then inserted up to about 2 mm proximal to the bifurcation of the PV. The cannula is secured with the proximal ligature, with which also the HA is tied off.

To prevent swelling of the liver by overfilling the circulation, the large abdominal vessels -aorta and VCIA- are cut beneath the caval ligature.

While the liver is flushed with oxygenated perfusate, the thorax is opened. With a pair of heavy scissors the thorax is cut in both midclavicular lines, and the diaphragm is trimmed away from the inferior surface of the sternum and both costal margins.

The thoracic inferior caval vein (VCIT) is bluntly freed from surrounding tissue and fat. A loose ligature is then laid around the vein. The heart is picked up by forceps and the wall of the right atrium is cut. A hooked polyethylene cannula (Portex Ltd.; OD 2 mm, ID 1.5 mm) is inserted from the entrance of the VCIT into the right atrium to just below the diaphragm, near to the inflow of the hepatic veins. The caval cannula is fixed by tieing the ligature. To direct the perfusate exclusively towards the caval cannula, the ligature around the VCIA is tied.

The diaphragm is retracted gently, and its leafs are dissected as close to the thoracic wall as possible. Then the stomach is retracted forward. This allows dissection of the oesofagus, the pancreas and the remainder of the gastro-hepatic ligaments.

The liver is now taken gently in one hand, while with the other hand still existing attachments of the liver to the body, including the crura of the diaphragm and several abdominal vessels, are divided.

Freed from all attachments, the liver is put on its support. To guarantee adequate flow, the liver is positioned with its hilus facing upwards, while the remaining leafs of the diaphragm are spread in such a way under the liver, that outflow obstruction is prevented.

Finally, the liver, laying on its support, is put in the organ chamber and connected to the perfusion apparatus. In this procedure the liver is devoid of oxygen for maximally five minutes. The whole procedure takes 15 to 20 minutes.

DISCUSSION

Liver function can be studied in the intact animal, the isolated perfused rat liver preparation, liver slices, cell cultures and homogenates, and in subcellular organelles.

The isolated rat liver preparation presents some important advantages. Primarily, this technique offers the opportunity to study liver function without interference of the action of other organ systems in the body, while the parenchymal and physiological integrity of the organ is not offended. Therefore, factors, that are directly concerned with the functioning of the organ can be controlled and manipulated easily. Furthermore, drugs and other substances can be administered in doses that would be letal for the intact animal.

In the model, presented here, perfusion is performed solely through the portal vein with an acellular perfusate under a constant pressure. The apparatus presents the following features:

1. The oxygen tension of the inflowing perfusate can be kept high, such that adequate oxygen delivery to the liver tissue is guaranteed.
2. The pH of the inflowing perfusate can be adjusted quickly and easily by changing the percentage carbon dioxide of the gas mixture with which the perfusate is gassed. Thus it is possible to equilibrate the acid-base balance within narrow (physiological) limits.
3. The perfusion characteristics are such that temperature induced changes in viscosity cannot hinder adequate perfusate flow through the liver.

4. Cooling and (re-)warming can be achieved quickly.

5. The cleaning protocol is simple, and prevented gross contamination in the present setup; only once in more than 60 perfusions growth of enterococcus has been observed. It should be noted, however, that for perfusions over six hours (Friedreich's period) asepsis should be aimed at.

It is obvious that the isolated rat liver preparation also has some disadvantages. An isolated liver is completely detached from metabolic and physiologic regulatory mechanisms, whereas for the unraffling of single biochemical processes the application of cell cultures and isolated cell organelles may be preferred. Nevertheless, isolated rat liver perfusion may prove to be an usable intermediate research technique between the "in vivo" and the "suborgan" techniques.

CHAPTER 3

MATERIALS AND METHODS

In this chapter the materials and methods, with general application to the chapters in which the experiments are described, are collected for the reader's convenience. Data of pertinent relevance to a particular chapter are presented there.

ANIMALS

Male outbred SPF rats were used throughout the experimental studies. The rats had free access to water and food (Hope Farms, Woerden, The Netherlands) till the moment of operation.

PROTOCOL; GENERAL REMARKS

For the experiments presented in chapters 4 and 5, 32 rats were used. They were subdivided into four groups of eight rats each. Their livers were, after hepatectomy, perfused hypothermically or normothermically: group A at 37°C, group B at 30°C, group C at 20°C and group D at 10°C.
Warm ischaemia time, defined as the period between clamping the liver vessels during hepatectomy and the start of the perfusion was in these groups always less than seven minutes.
For the experiments described in chapter 6, four new groups of eight livers were compared to the foregoing groups. These livers were, before the start of the perfusion, subjected to one hour normothermic non-flow anoxia. Group A2 was perfused at 37°C, group B2 at 30°C, group C2 at 20°C and group D2 at 10°C.
Each experiment consisted of a three hour period of perfusion at 10, 20, 30 or 37°C, followed by a fourth hour of perfusion at 37°C. This last hour of perfusion was incorporated in the

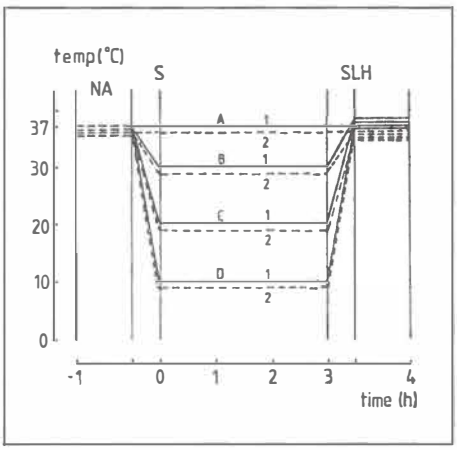


Fig. 3-1:The livers were divided into four groups; group A, perfused at 37°C, B at 30°C, C at 20°C, and D at 10°C.The groups A1,B1,C1, and D1 had a warm ischaemia time less than 7 minutes (solid line). The livers in groups A2,B2,C2, and D2 were subjected to one hour normothermic non flow anoxia prior to perfusion(broken line). After three hours of perfusion at 10,20,30 or 37°C, the livers were perfused a fourth hour at 37°C. S = start perfusion. SLH = start last hour of perfusion. N.A. = normothermic non-flow anoxia.

experimental protocol to study the reversibility of the effects of hypothermia.

The experiments started (Fig. 3-1) when the livers reached the desired temperature for the first three hours of perfusion. The last hour of perfusion started (SLH) when the livers had a temperature of 37 $\pm$ 1°C.

Cooling and rewarming over the largest temperature interval of 37 to 10°C *vice versa* took never more than 15 minutes.

In chapter 9 the same experimental protocol was used. This study concerned anoxia treated livers, perfused at 10 or 30°C, only.

ACID-BASE STATUS AND RESPIRATION

The acid-base status (pH; $cHCO_3^-$, mmol/l; P_{CO2}, kPa) of the perfusate was measured with an Acid Base Laboratory II (ABL II, Radiometer, Copenhagen). This apparatus combines pH, P_{CO2} and P_{O2} electrodes, and meets the criteria for accurate measurement of

the parameters as discussed by Severinghaus (178). Measurements were done at 37°C immediately after collecting the samples in plastic syringes of 1 ml. Calibration of the apparatus was performed according to the specifications of the manufacturer.

The $cHCO_3^-$ (mmol/l) was calculated by the apparatus according to the Henderson-Hasselbalch equation.

Oxygen consumption ($\dot{V}_{O2}$; ml/min x g dry weight) was calculated from the differences in P_{O2} between the in- and outflowing perfusate according to the following equation:

$$\dot{V}_{O2} = \alpha_{O2} (P^{o}_{O2} - P^{i}_{O2}) \times \dot{Q}^{p} / \text{g dry weight}$$

which α_{O2} denotes the solubility coefficient of oxygen in the perfusate (0.0003 ml/ml.kPa) and $\dot{Q}^{p}$ the perfusate flow (ml/min).

Carbondioxide production ($\dot{V}_{CO2}$; ml/min x g dry weight) was calculated from the differences in P_{CO2} and $cHCO_3^-$ between the in- and outflowing perfusate according to the following equation:

$$\dot{V}_{CO2} = ((6.P_{CO2} + 0.0506\ cHCO_3^{-o}) - (6.P_{CO2} + 0.0506\ cHCO_3^{-i})) \times \dot{Q}^{p} / \text{g dry weight}$$

in which 6 and 0.0506 denote transformation factors for calculating the CO_2 concentration in the out- (o) and in- (i) flowing perfusate.

The respiratory quotient (RQ) was calculated by dividing bicarbonate production by oxygen consumption:

$$RQ = \dot{V}_{CO2} / \dot{V}_{O2} .$$

BILE PRODUCTION

Bile production was measured every 30 minutes by weighing the bile, collected in preweighed plastic tubes. From these data, bile production per hour was calculated. To correct for differences in liver weight, bile production was expressed per g dry weight.

PERFUSION CHARACTERISTICS

The perfusate flow ($\dot{Q}^{p}$; ml/ min x g dry weight was measured directly by collecting the outflowing perfusate in a measuring glass during one minute. The perfusion pressure (P; kPa) was kept constant (0.13 kPa). From the $\dot{Q}^{p}$ and the P the vascular resistance (R; $N.s/m^{5}$ x g dry weight was calculated according to the analogue law of Ohm:

$$R = (Pi - Po) \times f / \dot{Q}^{p}$$

in which (Pi - Po) denotes the perfusion pressure and f a transformation factor. Since Po, the pressure in the outflowing perfusate, is measured in the hepatic vein, which drained freely in the perfusion system, this pressure is nihil. Therefore the perfusion pressure equals the hydrostatic pressure of the inflowing perfusate; P = Pi.

HISTOLOGY

Immediately after finishing the experiments, small blocks of tissue were taken from the left lateral, the median and the right

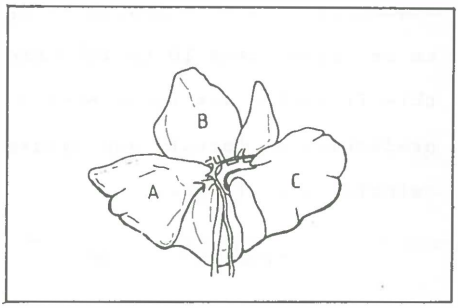


Fig. 3-2: Blocks of tissue were taken randomly from the left and right lateral (A and C) and the median (B) lobe for light microscopic examination.

lateral lobes for light microscopic examination (Fig. 3-2). The tissue blocks were fixed in formol 8% (pH 7.4), and stained in three ways; H.&E., Mallory and PAS after diastase digestion. In the microscopic examination of the tissue, attention was paid to sinusoidal architecture, signs of regression (e.g. necrosis) and steatosis.

WEIGHING AND DRYING

After finishing an experiment, the liver was freed of adjacent tissue, i.e. diaphragm and vessels. Then the liver tissue was weighed (wet weight). After drying overnight at 110°C the tissue was weighed again (dry weight). From these weights the dry/wet ratio was calculated.

LACTATE AND PYRUVATE PRODUCTION

Samples, taken from the in- and outflowing perfusate for the determination of the concentrations of lactate and pyruvate were deepfrozen immediately (-20°C). The concentrations were determined enzymatically aacording to Czok (179) and Gutman (180). In test series the relation between the lactate

concentrations of standard solutions and the extinction was found to be linear from 10 to 1000 mmol/l. For pyruvate concentrations this linearity was found over a range of 0 to 200 mmol/l.The production of lactate and pyruvate (mM/ h x g dry weight) was calculated as follows:

$$prod.(X) = 0.06 \times \dot{Q}^{p} \times (c(x)^{o} - c(x)^{i})/dry\ wt$$

in which X denotes lactate or pyruvate, and in which i and o refer to the in- or outflowing perfusate.

STATISTICS

For all measuring points, the data were grouped and the median (Md) and the range (R) were determined. Statistical analysis was performed two or one tailed, using Wilcoxon's two sample test. A difference was regarded significant for P 0.05.

SECTION III

APPLICATION OF THE MODEL

CHAPTER 4

THE EFFECT OF HYPOTHERMIA ON SOME FUNDAMENTAL PROPERTIES OF THE ISOLATED PERFUSED RAT LIVER

SUMMARY

The influence of cooling on vascular resistance (R), oxygen consumption ($\dot{V}_{O2}$), carbon dioxide production ($\dot{V}_{CO2}$), respiratory quotient (RQ), bile flow, and dry/wet weight of liver, was studied. Isolated rat livers were single pass perfused at 37, 30, 20 and 10°C (groups A,B,C,D) for three hours (n=8 in each group). To evaluate reversibility the livers were perfused during a fourth hour at 37°C.

$\dot{V}_{O2}$, $\dot{V}_{CO2}$, RQ, and bile production were lower in groups A and B than in groups C and D. There were no differences between A and B or between C and D. After rewarming, the differences disappeared. There were no differences in dry/wet ratio and the corrected vascular resistance. It is concluded that drastic alterations in metabolic and synthetic activity occur between 30 and 20°C. These can be explained by temperature induced alterations in membrane fluidity.

INTRODUCTION

Donor livers could, till the introduction of the "UW-solution" (158) be preserved safely up to about ten hours by storage on melting ice after being flushed with an intracellular type electrolyte solution (181) or a plasma solution (182). This relatively short preservation period limits the area of donor procurement, makes high demands upon the efficiency of transport arrangements, and has up to now prevented hepatic transplantation from becoming a truly elective operation (183).

Despite the very promising results with the "UW-solution", the exact nature of the limiting factors is unknown. In the knowledge that in profound hypothermia metabolic activity still exists (63), one can imagine that lack of nutritional support and accumulation of toxic metabolites play a crucial role.

Theoretically, it is likely that a change from simple cold storage to continuous perfusion, in which refreshment of the internal milieu can be guaranteed, leeds to prolongation of the preservation time for livers. Studies on continuous mechanical hypothermic perfusion of livers have thus far not shown that this technique is superior to simple cold storage (68).

It may well be assumed that profound hypothermia in itself is a principal factor in the deterioration of preserved livers. This assumption is substantiated by the finding that profound hypothermia may cause inactivation of the sodium pump, depletion of respiratory chain nucleotides, cellular swelling, membrane injury, and endothelial damage (68,184,185,186,187,188,189).

Although several investigators proposed the idea of preserving

livers and other organs at temperatures above 10°C (4,130,190), and although in normothermic ex vivo perfusions livers and kidneys remained vital for more than 48 hours (68,145,191), the functioning of isolated organs, in particular of the liver, at various levels of hypothermia never was a primary object of study.

Because knowledge on this subject may present clues to the prolongation of the preservation time of livers, the influence of cooling on several basic functional properties of the isolated perfused rat liver was studied in a single pass perfusion model.

MATERIALS AND METHODS

Thirty two male outbred Wistar rats, weighing 300 to 420 grams were subdivided in four groups (A,B,C,D; n=8 in each group). After hepatectomy their livers were perfused at 37 (A), 30(B), 20(C) and 10 (D)°C. The perfusion protocol was outlined in chapter 3.

The surgical technique, the perfusion apparatus, and the perfusate and its conditioning were described in chapter 2. The temperature of the livers was kept constant within a range of 2°C by means of the cooling and heating device.

Because the isolated liver is detached from the entero-hepatic circulation, bile salts are lost. Therefore sodium taurocholate (Calbiochem, La Jolla, USA; .025 umol/min) was infused continuously.

MEASUREMENTS

Perfusate flow, acid-base balance, oxygenation, and bile production were measured throughout the experiment.

The perfusate flow ($\dot{Q}^{p}$; ml/min . g dry weight) was measured directly at 0, 15, 60, 120, 180, and 240 minutes of perfusion. Vascular resistance (R) was calculated according to the analogue law of Ohm (chapter 3). To investigate if differences in R between the four groups were caused primarily by temperature induced changes in the perfusate's viscosity, the R of the livers in the different groups were standardized towards 37 C (R^{c} ; corrected vascular resistance). This was done by multiplying the calculated (actual) R by the ratio of the perfusate's viscosity at 37°C to the perfusate's viscosity at 10, 20 or 30°C.

The acid-base status -pH, $cHCO_3^-$ (mM), P_{CO2} (kPa)- and the oxygen tension (P_{O2} ; kPa) of the in- and outflowing perfusate were measured at 0, 15, 60, 120, 180 and 240 minutes. $\dot{V}_{O2}$, $\dot{V}_{CO2}$ and RQ were calculated as outlined in chapter 3.

Bile production was determined <u>in vivo</u> as well as during the experiments <u>(in vitro).</u>

To verify that all livers were comparable with respect to their bile production, bile was collected during 15 minutes before the hepatectomy was performed. Although bile production under these circumstances may be influenced by the opening of the abdominal cavity and by the manipulation of the abdominal organs, the <u>in vivo</u> bile production was regarded as a rough estimate of liver function.

RESULTS

PERFUSION DYNAMICS

Vascular resistance (R) was higher and perfusate flow ($\dot{Q}^{p}$) was lower in the groups D (10°C), C (20°C), and B (30°C) as compared to the normothermic group (37°C) during the first three hours of perfusion (Table 4-1; Fig. 4-1A). After correcting the the calculations for the resistance for temperature induced changes in the viscosity of the perfusate this picture changed entirely; the primary differences in resistance during the first

Table 4-1: Perfusate flow ($\dot{Q}^{p}$; ml/min x g dry weight) of rat livers in normo- and hypothermia (Median and Range). From t=0 to 180 minutes perfusion was performed at 37, 30, 20 and 10°C (A,B,C,D). From t=180 to t=240 minutes, all livers were perfused at 37°C. n=8 in each group.

	PERFUSATE FLOW AT VARYING TIME PERIODS OF PERFUSION (MIN) AT TEMPERATURES (°C) AS INDICATED						
GROUPS	Temp.	0′	15′	60′	120′	180′	Temp.	240′
A	37	13.12 8.80-18.26	13.27 10.40-19.57	14.12 12.96-19.52	13.82 12.22-20.87	13.41 12.69-20.87	37	12.92 10.74-15.65
B	30	11.99 8.33-17.28	11.99 8.95-14.80	11.80 8.95-15.20	11.78 8.42-15.20	11.99 7.37-16.00	37	12.21 7.37-16.80
C	20	9.29 5.13-16.25	8.43 8.13-16.88	9.08 8.26-15.63	9.89 7.83-15.63	9.68 7.83-15.63	37	11.10 8.70-13.13
D	10	7.48 5.00-12.30	9.22 6.06-12.30	9.76 6.06-14.61	9.09 6.76-12.30	8.88 5.46-12.30	37	13.34 8.85-17.30

three hours of perfusion disappeared (Fig. 4-1B). After rewarming there were no differences between the groups with regard to the uncorrected as well as the corrected vascular resistance.

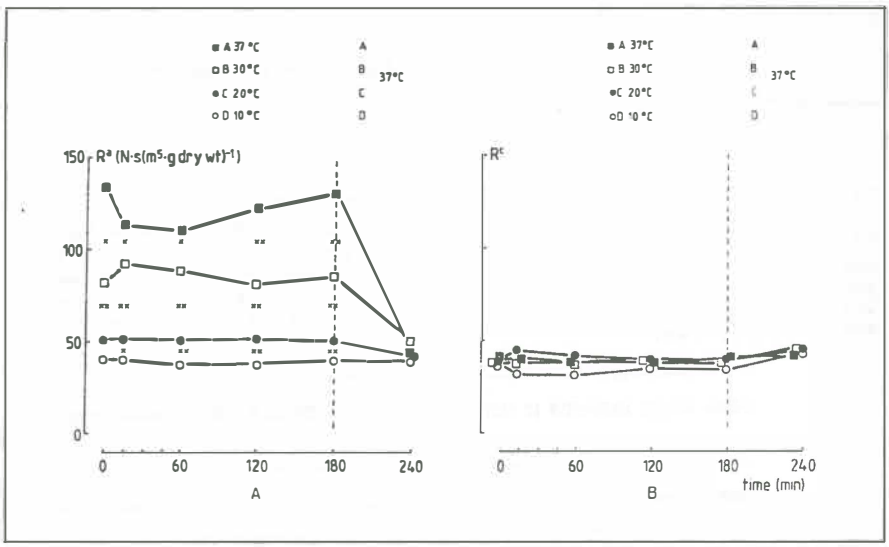


Fig. 4-1 A/B: Median uncorrected (A) and corrected vascular (B) resistance (R(actual) and R(corrected); N.s.m-5/ g dry weight). During the first three hours, livers were perfused at 37 (A;■), 30 (B;□), 20 (C;●) and 10°C (D;○). In the fourth hour all livers were perfused at 37°C. Data were analyzed by Wilcoxon's two sample test (n=8). * $0.05 > P > .01$; ** $0.01 > P > .001$.

RESPIRATION

Oxygen consumption ($\dot{V}_{O2}$), carbon dioxide production ($\dot{V}_{CO2}$), and the respiratory quotient (RQ) decreased with cooling in a nonlinear fashion. In the temperature interval between 30 and 20°C the decreases in $\dot{V}_{O2}$, $\dot{V}_{CO2}$, and RQ were about 50, 37 and 46 percent respectively. The decreases between 20 and 10°C, and

between 37 and 30°C were considerably less (Table 4-2; Fig. 4-2, 4-3).

After rewarming, most differences disappeared. Only the $\dot{V}_{O2}$ of group B was significantly lower than the $\dot{V}_{O2}$ of the groups C and D. Furthermore, the RQ of the livers in group D was significantly lower than the RQ of the livers in the other groups after rewarming.

Table 4-2: Carbon dioxide production ($\dot{V}CO2$; ml/min x g dry wt x 1000) of rat livers in normo- and hypothermia (Median and Range). From t=0 to 180 minutes, perfusion was performed at 37, 30, 20 and 10°C (A,B,C,D). From t=180 to t=240 minutes all livers were perfused at 37°C. P is the level of significance as calculated with Wilcoxon's two sample test. * .05>P>.01; ** .01>P>.001; *** P<.001. n=8.

CARBON DIOXIDE PRODUCTION AT VARYING TIME PERIODS OF PERFUSION (MIN) AT TEMPERATURES (°C) AS INDICATED								
GROUPS	Temp.	0′	15′	60′	120′	180′	Temp.	240′
A	37	.133 .110-.209	.174 .083-.318	.205 .108-.299	.194 .125-.281	.215 .108-261	37	.167 .096-.253
		*	*	**	**	*		*
B	30	.085 .005-.215	.111 .068-.150	.133 .105-.181	.140 .079-.198	.160 .093-.231	37	.255 .108-.329
		***	***	***	***	***		*
C	20	.024 .010-.037	.026 .019-.049	.035 .016-.053	.040 .016-.049	.037 .018-.060	37	.181 .115-.232
		*	*	*	*	*		
D	10	.010 .001-.130	.012 .004-.120	.015 .010-.170	.012 .007-.170	.015 .005-.080	37	.214 .138-.280

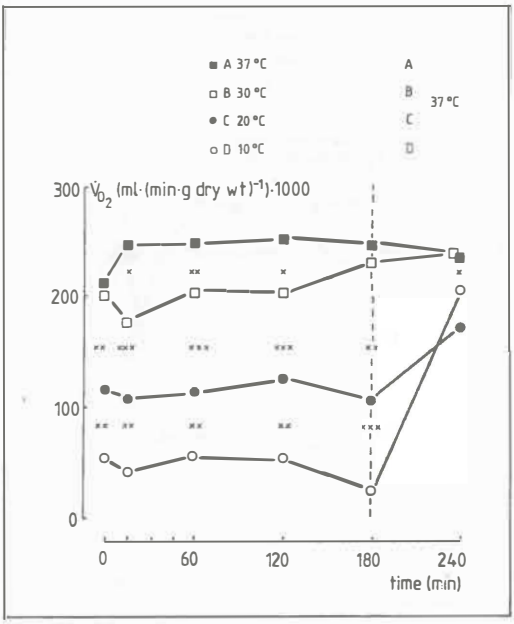
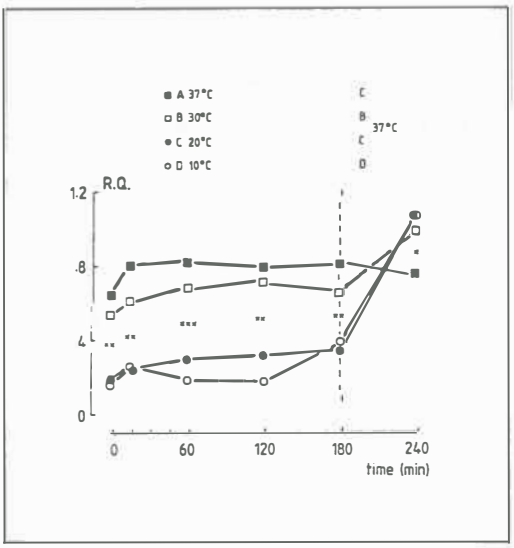


Fig. 4-2.: Median oxygen consumption (VO2; ml/(min x g dry weight) x 1000). Livers were perfused three hours at 37 (A;■), 30 (B;□), 20 (C;●) and 10°C (D;○), followed by a fourth hour at 37°C (n=8 in each group). Data were analyzed by Wilcoxon's two sample test. * .05>P>0.01; ** 0.01>P>0.001; *** P<.001.



4-3.: Median respiratory quotient (RQ). Livers were perfused at 37 (A;■), 30 (B;□), 20 (C;●) and 10°C (D;○) during three hours, followed by one hour perfusion at 37°C. Generally n=8. n=7; n=6. Data analysis was done by Wilcoxons two sample test. * 0.05>P>.01; ** 0.01>P>.001; *** P<0.001.

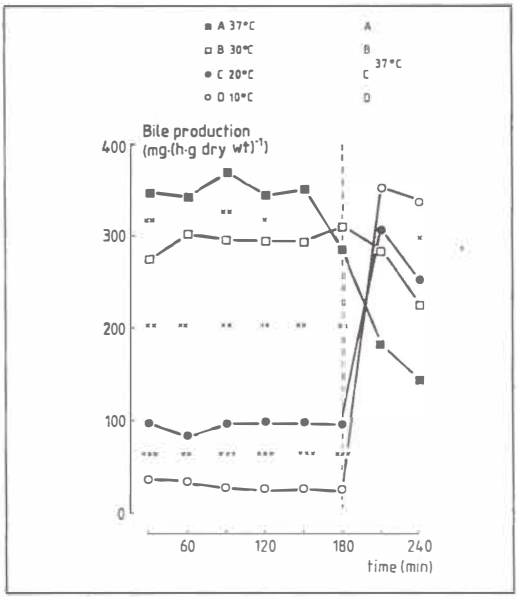


Fig. 4-4: Median bile production (mg/(h x g dry weight)) in normo- and hypothermia. Samples were taken every 30 minutes. Livers were perfused three hours at 37 (A;■), 30 (B;□), 20 (C;●) and 10°C (D;○), and a fourth hour at 37°C. n=8 unless indicated otherwise: n=7; n=6. Data were analyzed by Wilcoxon's two sample test.
* 0.05>P>0.01;
** 0.01>P>.001;
*** P<0.001.

BILE PRODUCTION

Bile production also decreased in a nonlinear manner with progressive cooling. Between 30 and 20°C bile production decreased with about 60 percent. Between 37 and 30°C, and between 20 and 10°C the decrease in bile production was about 15 and 20 percent respectively (Fig 4-4).

After rewarming the livers of group A had the highest bile production, whereas the livers in group D produced progressively less bile after 150 minutes of perfusion.

DRY/WET RATIO

The dry/wet ratios of the livers were the same in all groups after four hours of perfusion; group D 0.20/.15-.21 (Median/Range), group C 0.19/.12-.21, group B 0.16/.13-.19, group A 0.16/.13-.18.

DISCUSSION

Cooling causes marked alterations in the metabolic and synthetic activity of the isolated perfused rat liver. Oxygen consumption ($\dot{V}_{O2}$), carbon dioxide production ($\dot{V}_{CO2}$), the respiratory quotient (RQ), and the bile production decrease with cooling in a nonlinear relation with temperature. For all of them, a "break-point" appeared to exist between 30 and 20°C, which is probably a reflection of alterations in membrane structure and function, and in enzyme activity (see chapter 1).

$\dot{V}_{O2}$, $\dot{V}_{CO2}$, and, consequently, the RQ are related to the production of energy by the cell: glycolysis and oxidative phosphorylation. Oxygen and substrate are consumed only when adenosine diphosphate (ADP) is available in the mitochondrion to be converted to adenosine triphosphate (ATP) (24). Since the activity of the membrane bound enzyme adenine nucleotide translocase, which is responsible for the transport of cytosolic ADP across the mitochondrial membrane, is nonlinearly related to the temperature (24), the decrease in oxidative phosphorylation with the lowering of the temperature will be reflected in a nonlinear decrease in the $\dot{V}_{O2}$ and $\dot{V}_{CO2}$.
At the other hand, the transport of substrate (e.g. glucose) across the several cellular membranes may also show a nonlinear course during cooling. It has been shown in this connection that the transport of hexoses across the liver cell membrane shows a breakpoint in its activity at 18°C (192). When less oxidizable substrate is available, this subsequently will be reflected in a diminished $\dot{V}_{O2}$. Thus, when there is a break-point in the

transmembrane substrate transport, there will also be a break-point in the $\dot{V}_{O2}$ and the $\dot{V}_{CO2}$.
At first glance, it might be assumed that the reduction in $\dot{V}_{O2}$ will be proportional to the reduction in $\dot{V}_{CO2}$ in hypothermia, leaving the RQ unchanged. The present study shows, however, that also the RQ decreases with progressive cooling. In deep and profound hypothermia the RQ sank even to levels (about 0.3), that are never seen in normothermia. Although a decrease in the RQ has been noted before (193) - albeit not to such low levels - this phenomenon has never been explained.
One explanation may be found in the fact that cooling reduces the importance of glucose as a substrate for cellular (energy) metabolism. In normothermia glucose is the principle substrate for metabolism, resulting in a RQ of near to 1.0. In hypothermia, however, the combustion of other substrates as fatty acids is preferred over the oxidation of glucose (26,27,194), leading to a reduction of the RQ. This effect of cooling may be enhanced by the decrease in transmembrane transport of glucose (29,193) and the reduction of the activity of insulin (119,195,196) in hypothermia.
A second explanation may be related to a progressive inability of the cell to utilize oxygen with progressive cooling. The present study suggests that is becomes difficult for the cell to utilize oxygen in hypothermia as efficiently as in normothermia; more oxygen (i.e. a higher oxygen tension) is needed to produce the same amount of CO_2, leading to a decreased RQ. This suggestion is in line with the findings that cooling produces probably a shift in the balance between aerobic and anaerobic metabolism towards

the latter, and that relatively high oxygen tensions are needed to achieve aerobic (energy) metabolism in hypothermia (4).

The apparent breakpoint in the bile production may also be explained in two ways.

Firstly, the production of bile is (partly) energy dependent, thus requiring the presence of ATP. When the cellular energy production is reduced by cooling in a nonlinear manner, as described above, energy consuming processes, including bile production, consequently reflect this course.

Secondly, it is known that certain agents which decrease hepatocyte plasma membrane fluidity and Na^+/K^+-dependent adenosine triphosphatase activity (Na^+/K^+-ATPase) also induce cholestasis (197,198,199,200). It is in all probability the decrease in membrane fluidity which leads to the reduction in Na^+/K^+-ATPase activity, and to inhibition of the bile salt-dependent and -independent bile flow. Since membrane fluidity is also reduced by cooling, and because Na^+/K^+-ATPase activity shows a breakpoint around 25°C (201), the course of bile production during progressive cooling as found in this study may be explained by temperature induced changes in membrane structure and function. The increase in vascular resistance (R), as found in hypothermia, is probably caused by temperature induced alterations in the viscosity of the perfusate, since correction for these alterations largely eliminated the differences in R between the four groups.

Because no differences were found in the dry/wet ratio of the livers in the four groups, hypothermic perfusion of the liver with an acellular extracellular medium apparently causes no

oedema. This makes the use of the so called intracellular type of electrolyte solutions unnecessary in hypothermic perfusion preservation.

Most findings, e.g. the nonlinear relation between the variables and the temperature, can be explained from changes in enzyme activity and membrane structure and function. Temperature induced changes in membrane fluidity probably play a central role: By reducing the membrane fluidity cooling may affect the metabolic and synthetic activity of livers profoundly, thus limiting their maximum preservation time. Therefore, maintenance of optimal membrane fluidity may provide a clue towards prolongation of the preservation time of livers. This may be achieved by adding certain compounds which fluidize the cellular membranes (202). It seems, however, more rational to obtain optimal membrane fluidity by raising the preservation temperature, which presently is 10°C or less. The present study shows that this temperature should be between 20 and 37°C. It is suggested that further studies on long term liver preservation should be done at temperatures between 20 and 30°C (See also Appendix B).

CHAPTER 5

THE EFFECT OF HYPOTHERMIA ON LACTATE AND PYRUVATE PRODUCTION AND L/P RATIO AS MARKERS OF AEROBIC AND ANAEROBIC METABOLISM OF THE ISOLATED PERFUSED RAT LIVER

SUMMARY

The effect of cooling on the production of pyruvate and lactate and their production ratio (L/P ratio), as markers for aerobic and anaerobic glycolysis, was studied in the isolated perfused rat liver model. Pyruvate and lactate production in the 20 and 10°C groups were lower than in the 37 and 30°C groups during the first three hours of perfusion. Between the 20 and 10, and the 37 and 30°C groups were no differences. After rewarming these differences disappeared. The median L/P ratio was always about 6. It is concluded that cooling causes a reversible depression of aerobic and anaerobic glycolysis of the isolated perfused rat liver.

INTRODUCTION

Pyruvate and lactate are the most characteristic intermediates of aerobic and anaerobic glycolysis. Under aerobic conditions glycogen and glucose are transformed to pyruvate, which is further converted to water and carbon dioxide in the Krebs cycle under liberation of adenosine triphosphate (ATP).

In normothermia and normoxia, cellular and interstitial concentrations of pyruvate and lactate are in balance. This is expressed in the ratio lactate to pyruvate (L/P ratio), which normally remains below 10 (203,204). Since the concentrations of lactate and pyruvate are also in equilibrium with the concentrations of the redox couple $NAD/NADH_2$, the balance between NAD and $NADH_2$ is reflected in the more easily measurable L/P ratio (203). This can be summarized in the following equation:

$$c_l/c_p = 1/k\,(c_{NADH_2}/c_{NAD})$$

in which c denotes the concentration of lactate (L) or pyruvate (P), and k is a constant.

Hypothermia is the keystone of today's preservation techniques. It decreases and alters metabolism by reducing enzyme activity. In this respect, qualitative alterations in metabolism have been noted to change the balance between aerobic and anaerobic metabolism; in hypothermia relatively high oxygen tensions are needed to maintain aerobic energy transduction (4). Furthermore, the liver is separated from its hormonal and autonomic nerve control during in vivo preservation. It is obvious that this may cause marked alterations in the balance between aerobic and

anaerobic metabolism, even without cooling.
Thus, it is tempting to assume that the concentrations of pyruvate and lactate and their ratio may prove to be easily determinable parameters for metabolism and oxygenation during normo- and hypothermic liver preservation. It is also clear that during hypothermic liver preservation metabolism is affected and that criteria to describe the "normal" hypothermic state have to be defined.
Therefore, the effect of cooling on the production of pyruvate and lactate and the L/P ratio as markers for aerobic and anaerobic metabolism was studied in the isolated perfused rat liver model.

MATERIALS AND METHODS

Thirty two livers were perfused at 37, 30, 20 and 10°C (groups A,B,C and D; n=8 for each group) respectively.
Each experiment consisted of a three hour period of perfusion at one of the above mentioned temperatures. To evaluate the reversibility of the effect of cooling on the variables, the livers of groups A,B, and C were rewarmed and perfused for a fourth hour at 37°C. Thus, the livers in groups D were perfused four hours at 37°C (Table 5-1).
Oxygen tension and acid-base status of the inflowing perfusate and the perfusate flow were measured at 0, 15, 60, 120, 180 and 240 minutes of perfusion. Oxygen tension and acid-base balance were monitored with an Acid Base Laboratory II (Radiometer, Copenhagen). Perfusate flow was measured directly by collecting

Table 5-1: Experimental protocol. Groups A,B,C and D were perfused at 37, 30, 20 and 10°C respectively during three hours, followed by a fourth hour of perfusion at 37°C.

Group	3 hours perfusion at ..°C	1 hour perfusion at 37°C
A	37	yes
B	30	yes
C	20	yes
D	10	yes

the outflowing perfusate in a measuring glass for one minute. Pyruvate and lactate production and the L/P ratio were determined at 15, 60, 120, 180 and 240 minutes of perfusion as outlined in chapter 3.

For all measuring points data were grouped. Then the median and the range were determined for all groups. To show the relation between temperature and the production of lactate and perfusate by the livers in the four groups (A,B,C,D), Arrhenius plots, i.e. a plot of log (pyruvate) or log (lactate) against 1/T, were made. To study the effect of hypothermia on pyruvate and lactate production linear regression lines, connecting the medians of group A,B,C and D, were calculated.

Statistical analysis was done as outlined in chapter 3.

RESULTS

PYRUVATE PRODUCTION

Pyruvate production is non linearly related to temperature (fig.5-1, table 5-2); between 30 and 20°C the fall in pyruvate production was significantly greater than over the temperature intervals 37 to 30 and 20 to 10°C. This is also demonstrated by the Arrhenius plots, calculated from the pyruvate production (Fig. 5-2, Table 5-3). The median slope of the lines, connecting the medians of group B and C (0.077) is clearly steeper than the slopes of the lines, connecting A to B (0.037) and C to D (0.025).

After rewarming (t=240 minutes) pyruvate production in group A was lower than in groups B and C. There was a trend with respect to the difference between group A and D, indicating that livers in group A produced also less

pyruvate than the livers in group D after rewarming.

Table 5-2: Statistical comparison of the pyruvate production between groups A,B,C and D at 15, 60, 120, 180 and 240 minutes of perfusion. At t=240' all livers were perfused normothermically. The P values, presented here, were calculated with Wilcoxon's two sample test.

	time (min)				
Groups	15	60	120	180	240
C v. D	--	--	--	.07	.04
B v. D	.004	.001	.001	.001	.01
A v. D	.01	.001	.001	.001	.08
B v. C	.001	.001	.007	.004	--
A v. C	.01	.001	.01	.003	.1
A v. B	--	--	--	--	--

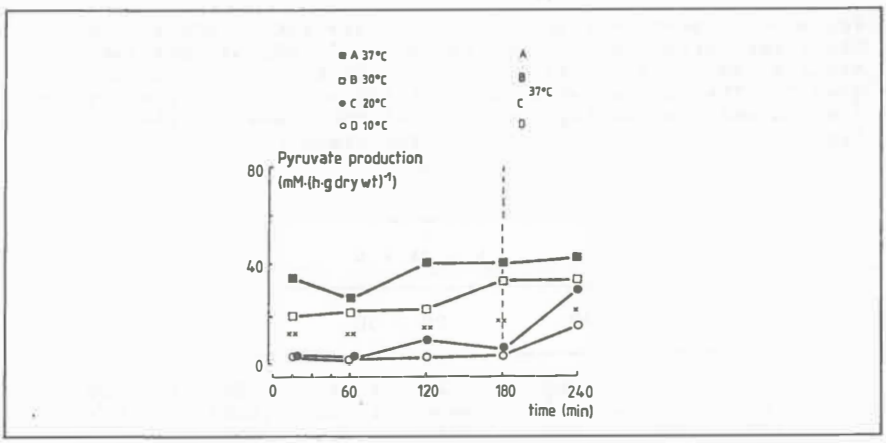


Fig.5-1: Median pyruvate production (mmol/h x g dry weight) of the livers in group A (■), B (□), C (●) and D (○). The livers were perfused during three hours at 37, 30, 20 and 10°C, followed by a fourth hour of perfusion at 37°C. For reader's convenience the medians of the four groups are given. Statistical analysis of differences was done with Wilcoxon's two sample test. * $.01 < P < .05$; ** $0.001 < P < .01$.

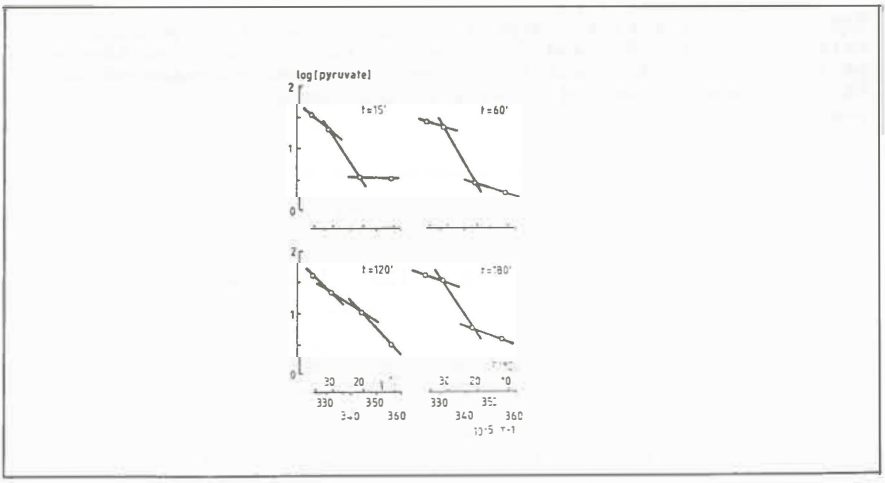


Fig. 5-2: Arrhenius plots of the pyruvate production at t=15, 60, 120 and 180 minutes of perfusion. There is a breakpoint in pyruvate production between 20 and 30°C.

Table 5-3:Linear regression lines of pyruvate production during the first three hours of perfusion (t=15, 60, 120 and 180 minutes) over the temperature intervals (dT) 10-20, 20-30, and 30-37°C. The general appearance of the regression lines is y=mx + n, in which m denotes the slope of the lines. In the bottom line the median slope (Md(m)) of the lines is put.

	y = mx + n		
t ±dT	10 - 20	20 - 30	30 - 37
15	.002x + .490	.077x - 1.010	.036 x + .329
60	.015x + .140	.090x - 1.360	.0147x + .911
120	.050x + .000	.034x + .320	.037 x + .226
180	.019x + .390	.076x - .750	.012 x + 1.144
Md(m)	.037	.077	.025

Table 5-4: Statistical comparison of the lactate production between groups A,B,C and D at 15, 60, 120, 180 and 240 minutes of perfusion. At t=240' all livers were perfused normothermically. The P values, presented here, were calculated with Wilcoxon's two sample test.

	time (min)				
Groups	15	60	120	180	240
C v. D	--	--	.1	.04	--
B v. D	.001	.001	.001	.001	--
A v. D	.001	.001	.001	.001	.1
B v. C	.001	.001	.001	.001	.08
A v. C	.001	.001	.001	.001	--
A v. B	.05	.1	.04	.07	.04

LACTATE PRODUCTION

Between 30 and 20°C lactate production decreased significantly stronger than between 37 and 30 and between 20 and 10°C (Fig. 5-3, Table 5-4). This finding is illustrated by the Arrhenius plots, calculated from the lactate production (Fig. 5-4, Table 5-5); the median slope of the lines connecting the median of group B and C during hypothermic perfusion (0.051) was clearly steeper than the median slope of the lines between A and B (0.020) and C and D (0.011).

After rewarming there was only a significant difference in lactate production between groups C and D; livers in group C produced more lactate.

Table 5-5: Linear regression lines of lactate production (t=15, 60, 120 and 180 minutes of perfusion) over the temperature intervals (dT) 10-20, 20-30 and 30-37°C. The general appearance of the lines is y=mx + n, in which m denotes the slope. In the bottom line the median slope (Md(m)) of the lines is mentioned.

	y = mx + n		
t ±dT	10 - 20	20 - 30	30 - 37
15	.008x + 1.330	.053x + .430	.040x + .820
60	.003x + 1.410	.072x - .090	.014x + 1.641
120	.026x + 1.100	.051x + .600	.016x + 1.650
180	.019x + 1.210	.046x + .670	.024x + 1.332
Md(m)	.011	.052	.020

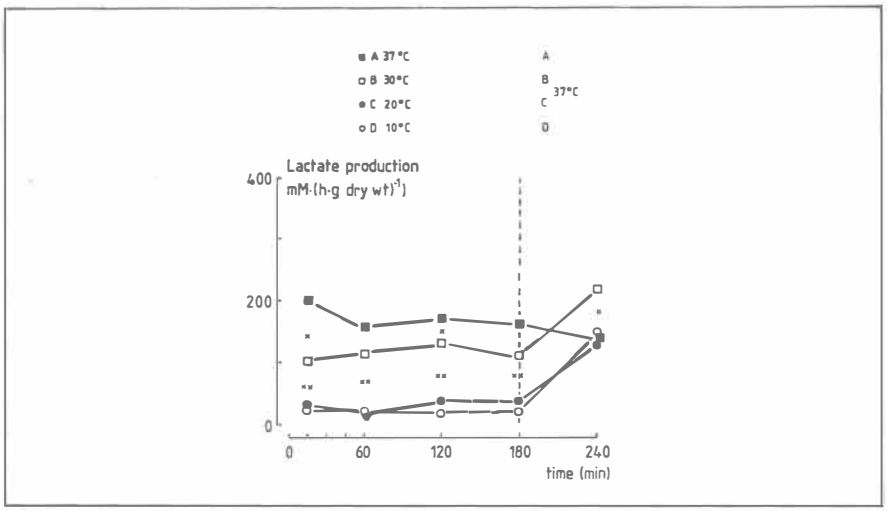


Fig.5-3: Median lactate production of the livers in group A (■), B (□), C (●) and D (○), perfused for three hours at 37, 30, 20 and 10°C, followed by a fourth hour of perfusion at 37°C. For reader's convenience the medians are presented. Differences between the groups were tested statistically with Wilcoxon's two sample test. * $.05 < P < .01$; $.01 < P < .001$.

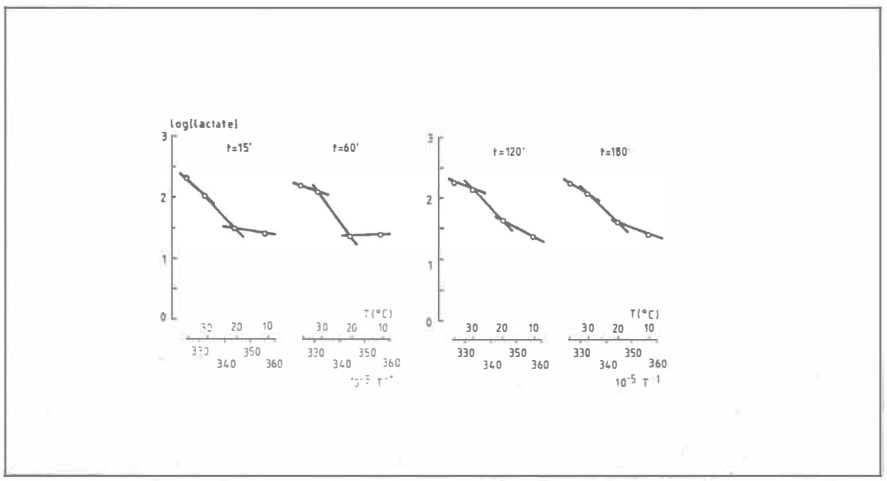


Fig. 5-4: Arrhenius plots of the lactate production of the livers in group A, B, C and D at 15, 60, 120 and 180 minutes of perfusion. There is an apparent breaking point in lactate production between 30 and 20°C.

L/P RATIO

The L/P ratio remained fairly constant (about 6) at all tempertures. There were no significant differences between the L/P ratio of the four groups.

DISCUSSION

There is a non linear relation between the production of pyruvate and lactate by isolated rat livers and temperature, whereas there are no differences in the L/P ratio of all these livers as well in normo- as in hypothermia.

The L/P ratio remained in the low normal range as compared to values given by other investigators (203,204). This may be caused by several factors. Firstly, the perfusate contained insulin and phenylalanin, both of which are known to lower the L/P ratio (203). Secondly, the isolated rat liver is detached from adrenal influence. From in vivo studies it is known that adrenalectomy reduces the L/P ratio (203).

The finding that the L/P ratios were the same in all groups at all temperatures may indicate that oxygenation of the isolated livers was always adequate, because NADH utilization is only optimal in the well oxygenated organ (205), leading to lower L/P ratios. Furthermore, this finding indicates that no major shifts in the balance aerobic/anaerobic metabolism occurred in the present study. This is consistent with reports, indicating that aerobic glycolysis is maintained in hypothermia only when high partial oxygen tensions - as was the case in the present study- are applicated (4).

Pyruvate production of hypothermically perfused rat livers always increased after rewarming. A remarkable finding, however, was that pyruvate production of livers perfused in profound hypothermia (10°C), stayed behind of the other groups. This may implicate that profound hypothermia depresses pyruvate production, i.e. aerobic glycolysis, such that restoration is impossible with rewarming, or that rewarming damages anaerobic glycolysis. Irrespective of the causal mechanism, this finding may also indicate that following perfusion in profound hypothermia uncoupling of metabolism occurs.

Pyruvate and lactate production deminished strongly over the temperature interval 30 to 20°C. Apparently there is a breakpoint in metabolic activity in this temperature range. A possible explanation for this observation is found in the non linear relation between the activity of certain enzymes and temperature (206). This may lead to a non linear cold induced reduction in the transport of substrates over membranes, and in the enzyme controlled transformation of these substrates. Furthermore, it has been shown that the transport of hexoses, e.g. glucose, accross the cellular membrane

In conclusion, cooling causes depression of aerobic and anaerobic glycolysis in isolated perfused rat livers. Except for the aerobic glycolysis of livers, perfused in profound hypothermia, this depression is reversible by rewarming towards normothermia. As to the preservation of livers for transplantation, this implies that livers, which can be perfused immediately following hepatectomy, i.e. within five minutes, with a well oxygenated medium, can be preserved at higher temperatures (e.g.in

normothermia) than the "conventional" 10°C, and that, regarding the influence of profound hypothermia on aerobic glycolysis, temperatures of 20°C and above should be preferred.

CHAPTER 6

HYPOTHERMIA AND THE FUNCTION OF ISOLATED PERFUSED RAT LIVERS WHETHER OR NOT SUBJECTED TO NORMOTHERMIC NON FLOW ANOXIA

SUMMARY

The effect of cooling and normothermic non-flow anoxia on the functioning of the liver was studied in the rat. Furthermore, the applicability of oxygen consumption ($\dot{V}_{O2}$), carbon dioxide production ($\dot{V}_{CO2}$), respiratory quotient (RQ), perfusion characteristics, bile production, pyruvate production, lactate production, and the L/P ratio as parameters of liver function in normo- and hypothermia was evaluated.

In the livers, pretreated with anoxia, bile production was lower in most experiments at all temperatures. In these livers pyruvate production was lower throughout the experiments in the 37 degrees group. There was a lower lactate production in all groups during the fourth hour of perfusion. $\dot{V}_{O2}$, $\dot{V}_{CO2}$, RQ, and perfusion characteristics showed no differences.

It is concluded that bile production is a good parameter for liver function in normo- and hypothermia and that hypothermic perfusion is needed to prevent damage to the aerobic glycolytic pathway of livers, treated with normothermic nonflow anoxia.

INTRODUCTION

All preservation methods, in current use, apply hypothermia to prevent the harmful consequences of anoxia, that inevitably occurs when organs are disconnected from their natural circulation. It is generally agreed that the protective effect of hypothermia is linked to the reduction of metabolic activity (31).

It is, however, also known that profound hypothermia in itself may exert negative effects on the condition of isolated organs. The inactivation of membrane pumps, resulting in cellular swelling and cell death, is a well known example of such an adverse effect (186,187,188).

In liver preservation it is furthermore known that, until the introduction of Belzer's "UW-solution (158), simple cold storage, which is generally used in all transplantation centers, and continuous hypothermic perfusion, used experimentally, did not bear possibilities of extending the safe preservation period of livers beyond eight to ten hours (68). Therefore, the possibility of preserving livers at less profound levels of hypothermia, at which the protective effect of hypothermia still exists and the adverse effects of more profound cooling are avoided, may be considered.

In anoxia, i.e. under anaerobic conditions, the cell has to do without the oxygen molecule, its most important hydrogen acceptor. In this situation, the transformation of NAD to $NADH_2$, which is essential for the continuation of glycolysis, continues because pyruvate takes over the role of oxygen as a hydrogen

acceptor. Thus, pyruvate is reduced to lactate. The accumulation of lactate causes intracellular acidification, which, in itself, has been shown to inhibit energy metabolism (207) as well as oxygen consumption of tissues (187).
In this respect it was stated recently that, besides the development of means to improve ex vivo preservation of the human liver also criteria should be established to evaluate its viability (208).
The present study concerns the effect of cooling on the functioning of isolated perfused rat livers whether or not subjected to normothermic non flow anoxia and with the assessment of easily determinable parameters for liver function during normo- and hypothermic perfusion.

MATERIALS AND METHODS

The livers of 64 outbred Wistar SPF rats,weighing 284 to 530 grams were subdivided into four main groups (A,B,C,D) and eight subgroups (A1, A2, B1, B2, C1, C2, D1, D2). They were treated and perfused as described in chapter 3. Surgical technique, perfusion apparatus, composition and conditioning of the perfusate, and perfusion technique were described in chapter 2.
Perfusate flow, acid-base status, oxygenation and bile production were measured throughout the experiment.
To evaluate if cooling prevents anoxic damage, and, if so, which level of hypothermia exerts the most beneficial effect, all groups of livers, that were treated with normothermic non flow anoxia (A2,B2,C2,D2) were compared with regard to the outcome of

several parameters at 240 minutes; i.e. after one hour normothermic perfusion.

The sampling scheme is given in table 4-1. Determinations and calculations as well as statistical analysis was done as outlined in chapter 3.

RESULTS

Tables of data are given in Appendix A.

PERFUSION DYNAMICS

Perfusate flow ($\dot{Q}^{P}$) decreased (Fig. 6-1) and vascular resistance (R) increased (Fig. 6-2) with lowering of the temperature. No difference was noted between the livers whether or not subjected to normothermic non-flow anoxia. During the fourth hour of perfusion, all differences between the groups A, B, C, and D, as seen during the first three hours of perfusion, disappeared.

RESPIRATION

Oxygen consumption ($\dot{V}_{O2}$), carbon dioxide production ($\dot{V}_{CO2}$) and respiratory quotient (RQ) of livers subjected and not subjected to normothermic non flow anoxia decreased non linearly with cooling (Figs. 6-3, 6-4, 6-5). There were generally no differences between the anoxia treated (A2, B2, C2, D2) and the other treated (A1, B1, C1, D1) livers. Only the livers of group C2 and D2 had a higher RQ during the first hour. The livers in group A2 had a lower RQ after 120 minutes of perfusion. After rewarming the livers in group D2 showed also a lower RQ.

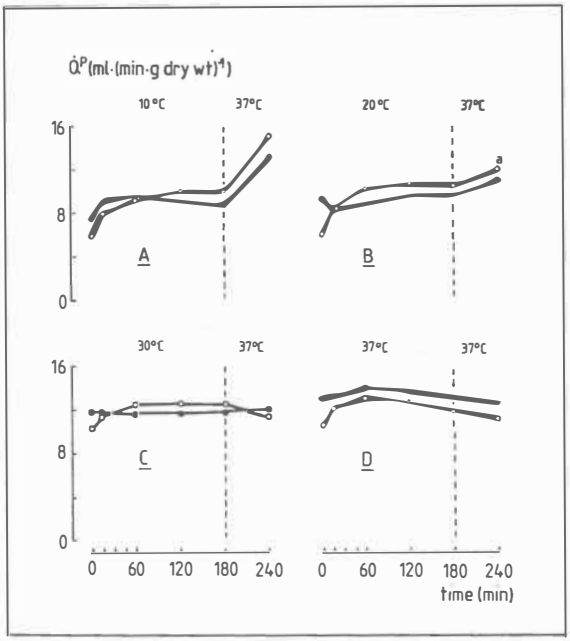


Fig.6-1: Median perfusate flow (Qp; ml/min x g dry weight) of livers subjected (O) or not subjected (●) to one hour normothermic non flow anoxia before perfusion at 37, 30, 20 or 10°C (groups A,B,C or D) for three hours, followed by a fourth hour of perfusion at 37°C. n=8, unless indicated otherwise: # n=7. There were no significant differences.

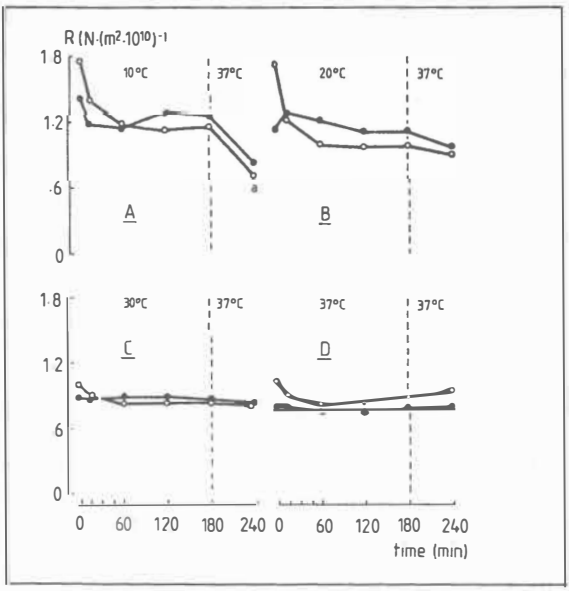


Fig 6-2:Median vascular resistance (R; Ns/m2 x 1000) of livers subjected (O) or not subjected (●) to one hour normothermic non flow anoxia, perfused for three hours at 37, 30, 20 or 10°C (groups A, B, C, D), followed by a fourth hour of perfusion at 37°C. n=8, unless indicated otherwise: #n=7.

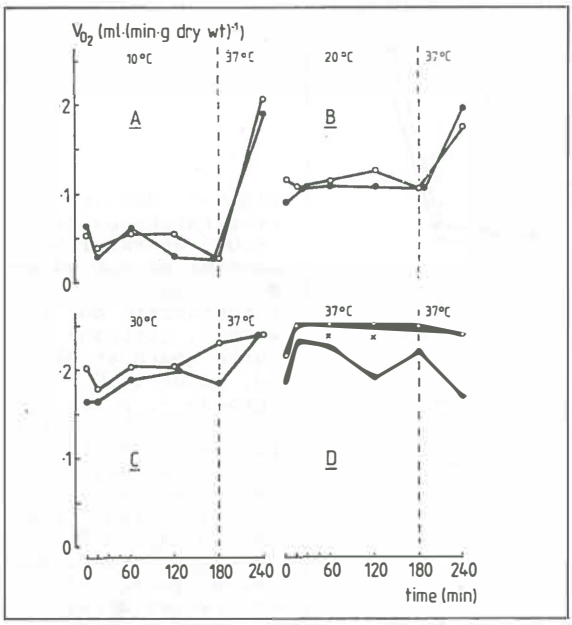


Fig.6-3: Median oxygen consumption (VO2: ml/min x g dry weight) of livers, whether or not (O or ●) subjected to one hour normothermic non flow anoxia, perfused at 37, 30, 20 or 10°C (groups A, B, C, D) for three hours, followed by a fourth hour of perfusion at 37°C. Statistical analysis was done with Wilcoxon's two sample test;* $P < .05$. n was always 8.

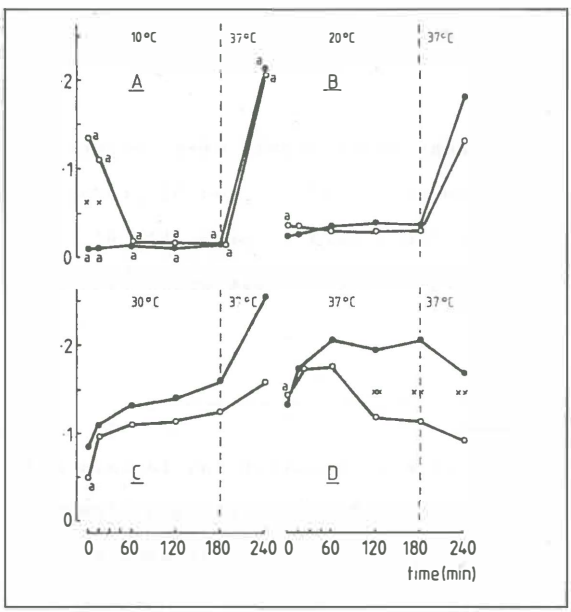


Fig.6-4: Median carbon dioxide production (VCO2; ml/min x g dry weight) of rat livers, treated or not treated (O or ●) with one hour normothermic non flow anoxia, pefused for three hours at 37, 30, 20 or 10°C (groups, A, B, C, D), followed by a fourth hour of perfusion at 37°C. Statistical analysis was done with Wilcoxon's two sample test.* $P < .05$; ** $.05 < P < .01$. n=8, unless indicated otherwise; # n=7.

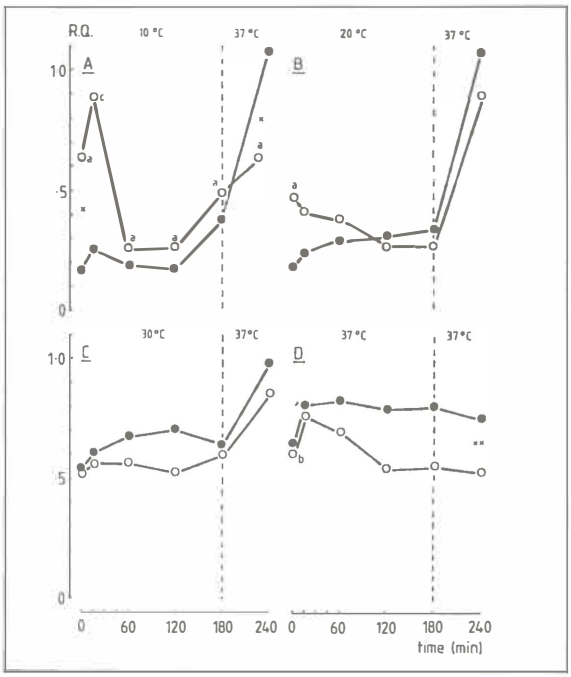


Fig.6-5: Median respiratory quotient (R.Q.) of rat livers, whether or not (O or ●) to one hour normothermic non flow anoxia, perfused for three hours at 37, 30, 20 or 10°C (groups A, B, C, D), followed by a fourth hour of perfusion at 37°C. Statistical analysis was done with Wilcoxon's two sample test:* $P<.05$; ** $0.05<P<.01$. n=8 unless indicated otherwise; # n=7, + n=6, $ n=5.

BILE PRODUCTION

Bile production showed a nonlinear relationship with temperature in all groups. The livers in groups A2, B2, C2 and D2 produced most of the time less bile than the livers in group A1, B1, C1 and D1 (Fig. 6-6). Only in groups C1 and C2 bile production was equal after rewarming.

PYRUVATE, LACTATE AND L/P RATIO

The relation between the production of pyruvate and lactate at the one hand and temperature at the other hand was non-linear (Figs.6-7, 6-8).Between 30 and 20°C the fall in pyruvate and

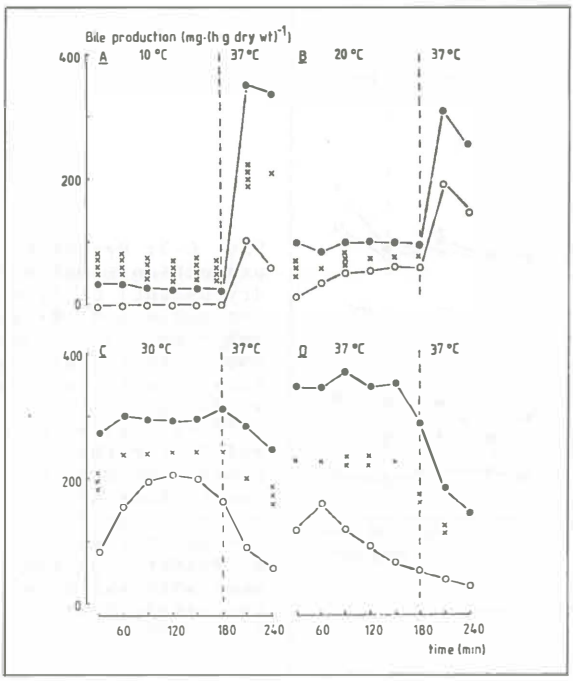


Fig. 6-6: Median bile production (mg/h x g dry weight) of livers that were not subjected (●) and livers that were subjected (O) to one hour normothermic non flow anoxia, perfused at 37, 30, 20 and 10°C (groups A,B,C,D) during three hours, followed by a fourth hour of perfusion at 37°C. Livers subjected to anoxia produced generally less bile (Wilcoxon's two sample test).
* $P < .05$; ** $P < .01$;
*** $P < .005$;
**** $P < .001$.
n was always 8.

lactate production was clearly greater than over the other temperature intervals. A remarkable finding was that only in group A (i.e. livers perfused normothermically for four hours) the anoxia-treated livers produced significantly less pyruvate throughout the experiment. In the other groups (B,C and D) there was no statistical difference (Fig. 6-7).

Lactate production showed clearly another picture (Fig.6-8). Anoxia treated livers produced only significantly less lactate after rewarming, i.e. after four hours of perfusion.

L/P ratio was always about 6. There were no differences between livers whether or not treated with anoxia (appendix A, table IX).

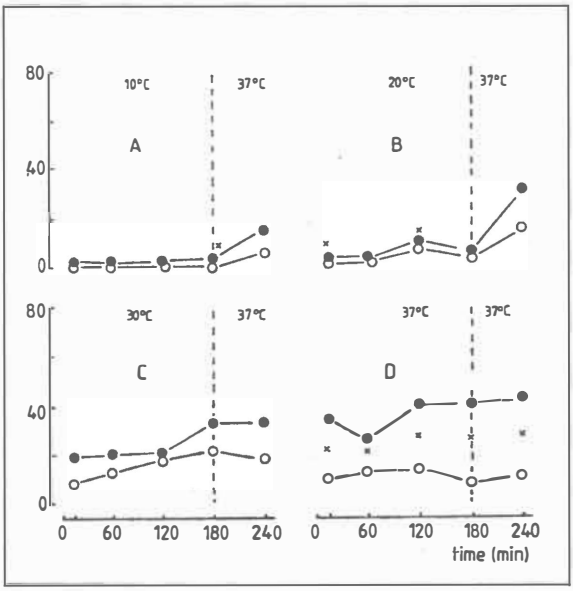


Fig. 6-7: Pyruvate production (mmol/h g dry weight) of livers not subjected (●) and subjected (O) to one hour normothermic non flow anoxia, perfused at 37, 30, 20 and 10°C (groups A, B, C and D) for three hours, followed by a fourth hour of perfusion at 37°C. Statistical analysis of differences was done with Wilcoxon's two sample test.
* $P < 0.05$.

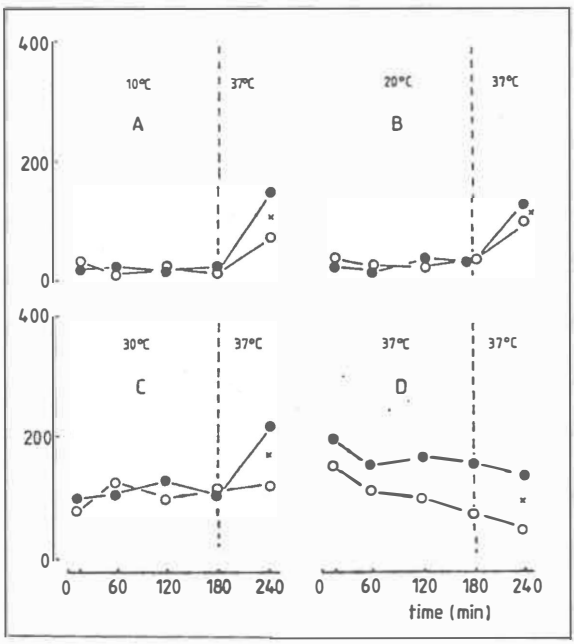


Fig. 6-8: Lactate production (mmol/h g dry weight) of livers not subjected (●) or subjected (O) to one hour normothermic non flow anoxia, perfused at 37, 30, 20 or 10°C (groups A, B, C and D) for three hours, followed by a fourth hour of perfusion at 37°C. Statistical analysis was done with Wilcoxon's two sample test.
* $P < 0.05$.

Table 6-1: P-values of mutual comparison of the livers that were treated with one hour normothermic non flow anoxia before perfusion for three hours at 37, 30, 20 or 10°C (groups A, B, C, D), followed by a fourth hour of perfusion at 37°C. The comparison was made after the fourth hour, i.e. one hour normothermic perfusion. Statistical analysis with Wilcoxon's two sample test. a or b indicates that the parameter in the first mentioned group is higher or lower than in the second mentioned group.

	D v.C	D v.B	D v.A	C v.B	C v.A	B v.A
Ox.cons.	--	--	--	--	--	--
Carbondiox. prod.	.03 b	.009 b	--	--	.04 a	.02 a
R.Q.	.05 b	.07 b	--	--	.005 a	.006 a
Vascular resist.	.05 b	--	.08 b	--	--	--
Bile prod.	.06 b	--	.09 a	.009 a	.005 a	--
Lactate prod.	.01 b	.009 b	--	--	.03 a	.01 a
Pyruvate prod.	.1 b	.009 b	.01 b	--	--	--
L/P ratio	--	--	.04 a	--	--	.1 a
Dry/wet ratio	--	.1	.01 a	--	.01 a	.004 a

DRY/WET RATIO

There were no differences in dry/wet ratio between the anoxia treated and the not anoxia treated livers of the same temperature group; i.e. A1 v. A2, B1 v. B2, C1 v. C2, D1 v. D2 (appendix A, table X).

HYPOTHERMIA AND ANOXIA

Carbon dioxide production, RQ and lactate production was lower in group D2 (10°C), than in group C2 (20°C) and group B2 (30°C), whereas the livers in group C2 and B2 had a higher carbon dioxide production, RQ and lactate production than the livers in group

A2. Bile production in groups C2 and D2 was higher than bile production in group A2, while group D2 produced less bile than group C2. Pyruvate production of the livers in group D2 was lower than in all other groups. The dry/wet ratio of groups B2, C2 and D2 was clearly higher, than the dry/wet ratio of group A2 (Table 6-1).

DISCUSSION

Metabolic and synthetic activity of isolated perfused rat livers, whether or not subjected to normothermic non-flow anoxia, dereased in a similar fashion with cooling, while bile production showed to be a good parameter for liver viability in normo- and hypothermia.

There were no major differences in $\dot{V}_{O2}$ and $\dot{V}_{CO2}$ between the livers that were or were not subjected to normothermic non-flow anoxia. However, the RQ of anoxia-treated livers, continuously perfused at 37°C, was relatively low. Obviously, anoxia induces changes in the subcellular handling of oxygen. Most likely these alterations occur at the mitochondrial level, since it has been shown that hypoxia reduces the rate of oxygen supply to mitochondria (209). From the present results it is clear that $\dot{V}_{O2}$, $\dot{V}_{CO2}$ and RQ are no suitable parameters for liver viability.

Bile production has been used by many investigators as a parameter of liver function during isolated perfusion (210) although its applicability has been disputed (211). The present study shows, however, that bile production is a reliable parameter, indicative for (anoxic) injury of the liver in normo-

and hypothermia. Although maybe less sensitive than other viability parameters (210), measuring of the bile production bears a distinct advantage by its simplicity.

Because there were no differences in dry/wet ratio between the anoxia-treated and the not-anoxia-treated livers, it may be concluded that no oedema had formed. The abscence of differences in perfusion characteristics may indicate that the so called "no reflow phenomenon", well known in kidney preservation (212), did not appear.

When anoxia treated livers were compared to livers that were not treated with normothermic non-flow anoxia, pyruvate production showed only significant differences between the groups that were continuously perfused at 37°C. In all probability, the anoxic damage to the aerobic glycolysis occurs immediately after reperfusion at 37°C, because the anoxia treated livers showed a lower pyruvate production from the beginning of the perfusion. This may point at the so called reperfusion phenomenon, which may be seen when previously anoxic tissue is reperfused with an oxygen rich medium (213,214). There were no such differences when all hypothermically perfused livers were rewarmed and perfused normothermically during a fourth hour. Apparently cooling counteracts the detrimental effects of anoxia on aerobic glycolysis.

Lactate production by livers, not treated with anoxia, was always higher after four hours of perfusion, i.e. after rewarming (groups B,C and D) and after the fourth hour of normothermic perfusion (group A). This may indicate that these livers are capable of releasing accumulated lactate more efficiently and

that there is temperature dependency regarding lactate production and release. Obviously there is also a time dependency, since the livers that were continuously perfused normothermically (group D), only brought about the difference in lactate production after four hours of perfusion.

From the mutual comparison of the anoxia treated livers after one hour normothermic perfusion several interesting conclusions can be drawn.Bile production in the 10 and 20°C groups was better than in the other groups, whereas the 20°C group was slightly better than the 10°C group. Furthermore, pyruvate production of the 10°C group was lower than of the other groups. These data may lead to the conclusion that in particular 20°C is an ideal temperature for preservation of principle processes as bile production and aerobic glycolysis. The fact that livers, that were perfused hypothermically initially had a higher dry/wet ratio than the livers that were perfused normothermically continuously is indicative for a distorted cellular volume regulation and may be seen as an argument in favor of hypothermically perfusion.

With regard to liver preservation, the findings regarding aerobic glycolysis, i.e. pyruvate production, indicate that livers that suffered from a long period of normothermic anoxia should be preserved in mild to profound hypothermia. When the data on bile production of anoxia treated livers are taken into account also, there might be a slight preference for preservation at 20°C. However, livers that can be perfused immediately following hepatectomy may possibly even be preserved under normothermic conditions.

CHAPTER 7

HISTOPATHOLOGY OF ISOLATED PERFUSED RAT LIVERS WHETHER OR NOT SUBJECTED TO NORMOTHERMIC NON FLOW ANOXIA

SUMMARY

Isolated rat livers, whether or not subjected to one hour normothermic non-flow anoxia, and perfused for three hours at 10,20,30 or 37°C, followed by a fourth hour of perfusion at 37°C, were examined with regard to changes in macroscopic and light microscopic appearance. The results showed that anoxia perse does not produce histopathological changes. However, one hour anoxia followed by perfusion at any temperature causes marked hepatocellular and vascular damage. Optimal results were obtained if initial anoxia was omitted and the livers were perfused at 10°C.

INTRODUCTION

The isolated perfused rat liver model has been used extensively for studies on hepatic function (e.g. 173, 174,175). Although degenerative changes have been observed (215), it proved an ideal preparation to study "normal" metabolism under normothermic conditions. On the contrary, experience with isolated rat livers, perfused at subnormal temperatures is, however, very scarce. In the preservation of organs, e.g. liver for transplantation, better insight into functioning under hypothermic conditions may eventually improve the outcome of preservation and transplantation (4).

In liver preservation, as it is presently done, the liver's morphological integrity is threatened in at least two ways, notably normothermic anoxia during donor hepatectomy and the hypothermia itself.

With regard to perfusion preservation, it is of interest to investigate at which moment of the preservation process the morphological damage arises: 1. during the (normothermic) anoxic period, 2. at the beginning of perfusion, or 3. in the course of the perfusion. It is, furthermore, important to study whether hypothermia indeed protects against the detrimental effects of normothermic non flow anoxia and if so, which level of hypothermia is most protective.

The aim of the present study was to examine the changes in macroscopic and light microscopic appearance of isolated rat livers, whether or not subjected to normothermic non-flow anoxia, normo- or hypothermically perfused with a hemoglobin free medium.

MATERIALS AND METHODS

A total of 71 livers from non fasted male Wistar rats, weighing 270 to 530 grams, of which 64 were mentioned in chapter 6, was used. The experimental protocol is outlined in Table 7-1.

Table 7-1: Overview of the experimental protocol.

GROUP	n	1 HOUR ANOXIA	3 HRS PERF.AT .. °C	4th H. PERF.AT 37 °C
A1	8	no	37	yes
A2	8	yes	37	yes
B1	8	no	30	yes
B2	8	yes	30	yes
C1	8	no	20	yes
C2	8	yes	20	yes
D1	8	no	10	yes
D2	8	yes	10	yes
Contr.	7	yes	no	no

Thirty two livers were, within seven minutes after hepatectomy, perfused in an unidirectional manner through the portal vein with modified Eagles medium in a balanced salt solution (chapter 2) at 37 (group A1), 30 (group B1), 20 (group C1) or 10°C (group D1) during three hours, followed by a fourth hour of perfusion at 37°C. Another 32 livers were subjected to one hour normothermic-non-flow-anoxia before perfusion at 37, 30, 20 and 10°C (groups A2, B2, C2, D2) during three hours and a fourth hour at 37°C. Seven livers (controls) were subjected to one hour normothermic-

non-flow-anoxia, after flushing with the perfusion medium. They were not perfused.

The livers were examined macroscopically with regard to color changes, oedema and changes in consistency.

For light microscopical examination small blocks of liver tissue (about 0.5 x 0.5 x 0.25 cm) were taken randomly from the left and right lateral lobes and the median lobe of the liver. The blocks of liver tissue were fixed in 8 % paraformaldehyde in phosphate buffered saline (PBS; pH 7.4). The fixed tissue blocks were dehydrated and embedded in paraplast. Staining methods on sections of 4 um thickness included hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) following diastase digestion and Mallory.

RESULTS

MACROSCOPIC EXAMINATION (Table 7-2)

Macroscopic alterations were most frequently seen in livers, subjected to anoxia and livers, continuously perfused at 37°C. There was no clear difference in frequency of discoloration between livers perfused at different temperatures. All livers, except for those in the control group, showed a livid/pale brown discoloration at the point of contact with the support in which they were positioned during perfusion. Regarding consistency the livers in group C (20°C) showed no alterations. Oedema was seen sporadically. Livers in the control group showed no macroscopic alterations at all.

Table 7-2: Macroscopic findings of livers, whether (2) or not (1) subjected to one hour normothermic non flow anoxia, and perfused at 37 (A), 30 (B), 20 (C) or10°C (D) for three hours, followed by a fourth hour of perfusion at 37°C. Control livers were only subjected to one hour normothermic non flow anoxia. The numbers (n) refer to the number of livers showing a particular alteration.

	D		C		B		A		
	1	2	1	2	1	2	1	2	cont.
Normothermic non flow anoxia	-	+	-	+	-	+	-	+	+
Perfusion temperature before rewarming (°C)	10		20		30		37		-
Number(n)	8	8	8	8	8	8	8	8	7
DISCOLORATION									
peripheral palor	2	4	0	3	1	2	2	3	0
irregularly distributed pale mottling	0	2	0	4	0	3	3	4	0
reticulated fine mottling over the entire liver surface	0	1	0	0	0	0	0	0	0
Total number	2	7	0	7	1	5	5	7	0
ALTERATIONS IN CONSISTENCY									
induration	0	3	0	2	0	0	2	2	0
softness	0	2	0	0	0	0	0	0	0
Total number	0	5	0	2	0	0	2	2	2
ALTERATIONS IN "WATER BALANCE"									
oedema	0	3	0	2	0	0	0	2	0
"transudate"	0	0	0	0	0	0	0	0	0
Total number	0	3	0	3	0	0	0	2	0
Total number with macroscopic alterations	2	8	0	8	1	5	7	8	0

LIGHT MICROSCOPIC EXAMINATION (Table 7-3)

Not anoxia treated plus perfusion

Most livers, not treated with one hour normothermic non-flow anoxia, showed steatosis, predominantly in zones 2 and 3 (Fig. 7-

Table 7-3: Light microscopical findings in livers, whether (2) or not (1) subjected to one hour normothermic non flow anoxia, and perfused at 10 (D), 20 (C), 30 (B) or 37°C (A) for three hours, followed by a fourth hour of perfusion at 37°C. Control livers were only subjected to one hour normothermic non flow anoxia. BHN = bridging hepatic necrosis.

	D		C		B		A		
	1	2	1	2	1	2	1	2	cont.
Normothermic non flow anoxia	-	+	-	+	-	+	-	+	+
Perfusion temperature before rewarming (°C)	10		20		30		37		
Number	8	8	8	8	8	8	8	8	7
HEPATOCELLULAR ALTERATIONS									
steatosis (large droplet)	6	6	8	8	4	8	3	4	4
rounding of cells	0	2	0	0	0	0	3	2	0
regression	2	6	5	4	2	3	4	7	3
necrosis	0	8	5	4	2	3	5	7	0
BHN	0	2	0	3	0	3	4	4	0
PORTAL ALTERATIONS									
infiltrate	0	3	0	3	0	3	3	3	0
oedema	0	2	0	0	1	3	1	0	0
SINUSOIDAL ALTERATIONS									
congestion	4	2	3	3	2	3	7	3	0
INTRAVASCULAR ALTERATIONS									
coagulation	1	0	0	0	0	1	3	3	0
CHOLESTASIS	0	0	0	0	0	0	0	0	0
Total number with microscopic alterations	6	8	8	8	5	8	5	7	4

1). In the livers, perfused initially at 30°C or lower temperatures relatively mild alterations were predominantly seen in zone 3 and to a lesser extend in zone 2. BHN was never seen in these livers. Livers in group D1 (10°C) showed even only little regressional changes. Livers in group A1 (37°C) however, had

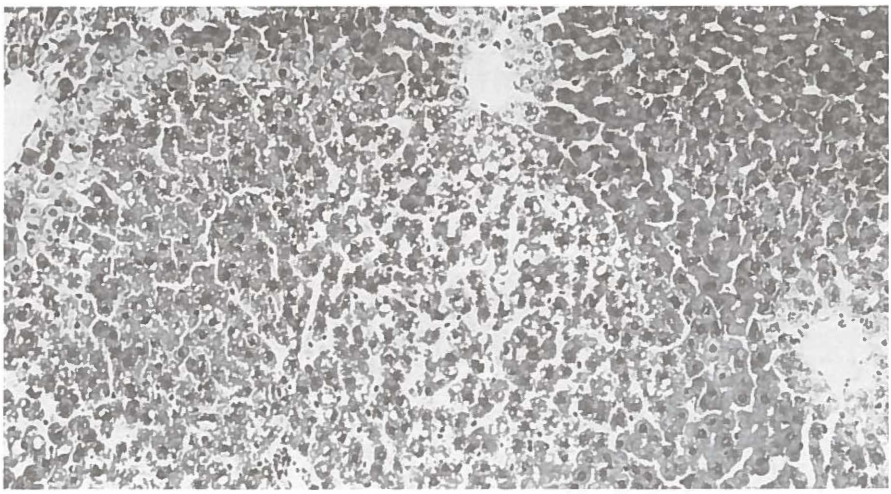


Fig. 7-1: Liver, not treated with anoxia, perfused at 37°C during four hours. There is slight peri-portal and acinar steatosis. Liver cell necrosis is notably absent (H&E, x200).

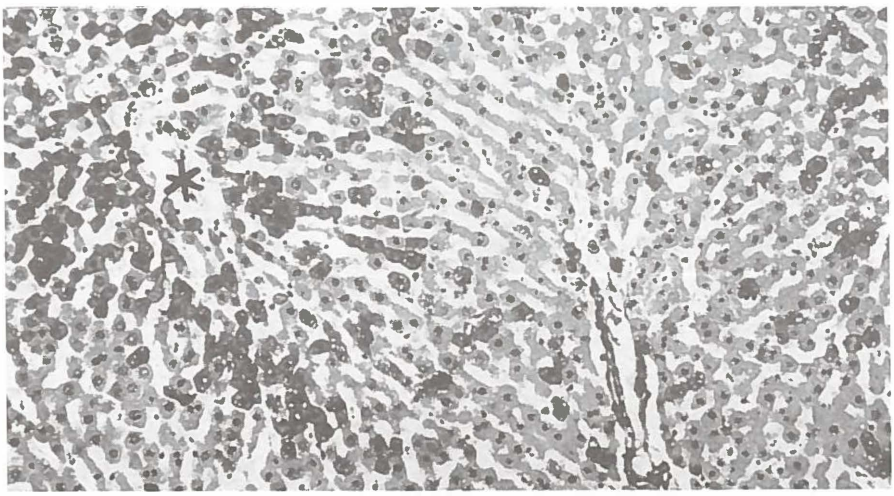


Fig. 7-2: Liver, treated with one hour normothermic-non-flow-anoxia prior to four hours of perfusion at 37°C. There is massive necrosis of hepatocytes. In the portal tracts (asterisk) single liver cells still appear vital (H&E, x200).

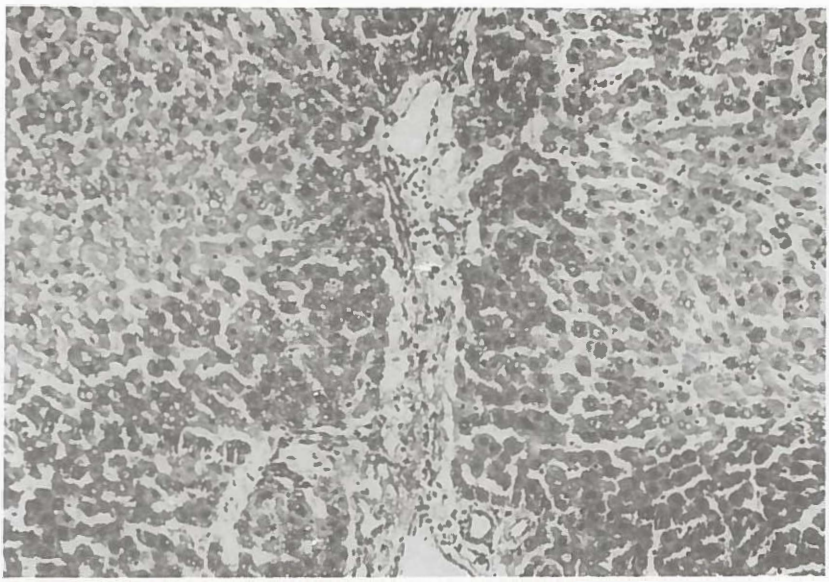


Fig. 7-3: Biopsy of a liver, treated with 60 minutes of normothermic-non-flow-anoxia before perfusion at 37°C during four hours. Necrosis of liver cells in the acinar zone 3. The hepatocytes in the periportal areas have a normal appearance (H&E, x200).

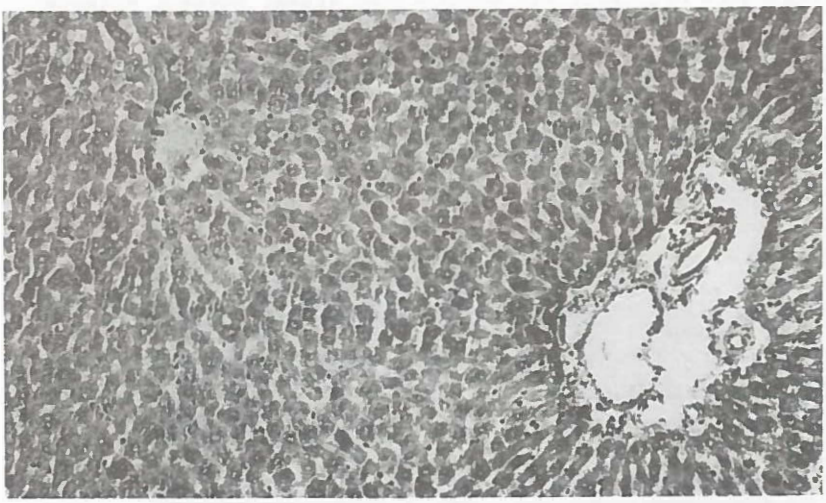


Fig. 7-4: Biopsy of a liver, not treated with anoxia, perfused for three hours at 20°C, followed by a fourth hour of perfusion at 37°C. The histologic appearance of this liver is nearly normal (H&E, x200).

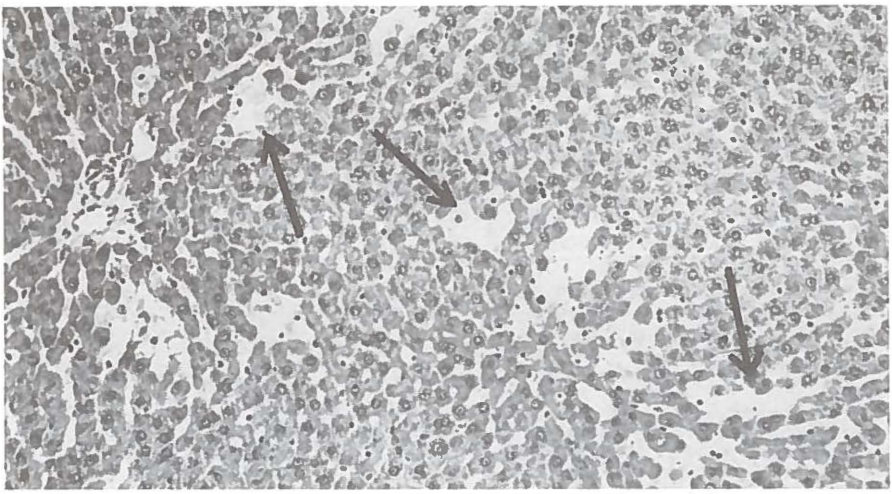


Fig. 7-5: Liver, anoxia treated and perfused at 30°C during three hours, followed by a fourth hour of normothermic perfusion. Randomly throughout the liver parenchyma peliosis-like areas lacking liver cells are present (arrows) together with widening of sinusoids. Otherwise the hepatocytes appear normal, without signs of degeneration and necrosis (H&E, x200).

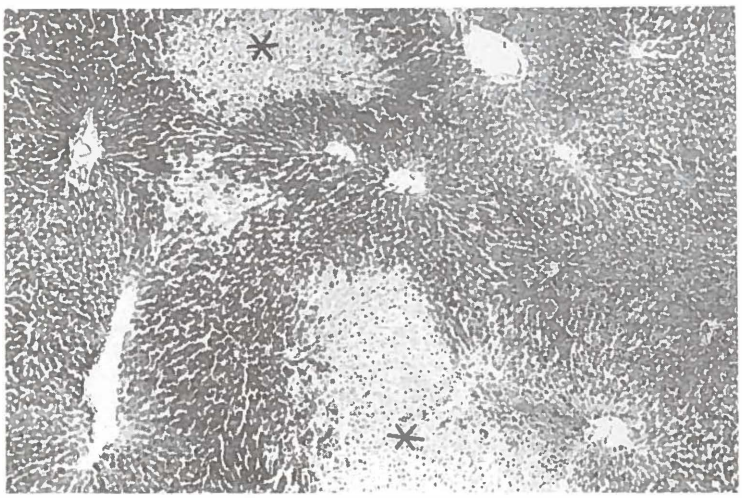


Fig. 7-6: Liver, not anoxia treated, perfused during four hours at 37°C. Random areas with liver cell necrosis (asterisks) are present throughout the liver parenchyma (H&E, x80).

Fig. 7-7: Liver, treated with one hour normothermic-non-flow-anoxia prior to four hours of perfusion at 37°C. Massive liver cell necrosis. Only in the subcapsular area (arrow) liver cells still have a normal appearance (H&E, x80).

important hepatocellular alterations including BHN (Fig. 7-6). Portal, sinusoidal, intravascular alterations and cholestasis were hardly or not seen (Fig. 7-4).

Anoxia treated plus perfusion

The livers treated with one hour normothermic non-flow anoxia showed severe hepatocellular alterations. predominantly in zones 2 and 3, ranging from rounding of cells to extensive BHN (Figs. 7-2, 7-3 and 7-7). Portal infiltration, portal oedema and congestion were also seen in these livers. There was only little difference between the four groups, perfused at different temperatures. Only groups B and C (30 and 20°C) showed a little

less hepatocellular degeneration (Fig. 7-5).

Anoxia treated, not perfused

Except for mild steatosis and regression in zone 3, the livers in the control group showed no important hepatocellular or other histological alterations.

For the overview of all data one is referred to Table 7-3.

DISCUSSION

The present study and experimental protocol were designed to test the histopathological effects of one hour anoxia perse (control group) and of three hours perfusion at various temperatures with our without preceeding normothermic non flow anoxia. The results demonstrate, that one hour anoxia perse does not produce histopathologic alterations, but that one hour anoxia followed by perfusion of the liver at any temperature produces marked hepatocellular and vascular damage. Optimal results were obtained if the initial normothermic non flow anoxia was omitted and the livers were perfused at 10°C (table 7-3: group A1). In general, it appeared that hepatocellular and circulatory alterations were more marked and irreversible at higher temperatures up to 37°C. It is generally accepted that the liver cell is highly vulnerable to anoxia (68) and that cooling may prevent its deleterious effects (31). Lack of oxygen initiates a chain of events, including inhibition of the synthesis of ATP (216,217), accumulation of inorganic phosphate (218) and lowering of the intracellular pH (207). Ultimately this may lead to cell death (194). After a period of anoxia, the subsequent reoxygenation

during perfusion plays a crucial role in the progression of these deleterious effects to morphologically detectable alterations of the liver cell injury. This is in line with the findings of other investigators (212) and comparable to the "perfusion paradox", observed following reperfusion of previously anoxic cardiac tissue (213). Here, reperfusion of anoxic tissue aggravates tissue injury and disturbes cell volume regulation. The complex process of degenerative changes following perfusion, known as the "reperfusion phenomenon", is thought to be related to the deleterious effects of anoxia, enhanced by oxygen free radicals, influx of calcium ions and energetic processes (212,214). Johnson, and Hearse et al proposed that as well the influx of calcium ions as the production of oxygen free radicals might play a role in the apearance of the "reperfusion phenomenon" (212,214).

Apart from anoxia, also a prolonged period of hypoxia during perfusion may lead to cellular damage. This is illustrated by the livers in group D1, that were treated with four hours normothermic perfusion without preceeding anoxia. In these livers, the pathologic alterations were primarily seen in the acinar zones 2 and 3, known to be vulnerable to hypoxia. From the results of this study it can be conjectured that the alterations following perfusion hypoxia need more time to develop and have another distribution throughout the liver parenchyma than those seen in the normothermic non flow anoxia livers following subsequent perfusion.

The protective effect of hypothermia on the occurrence of cell injury after normothermic non flow anoxia was in the present

study only minimal. There was a tendency that livers, perfused at 10°C showed less degenerative changes. Probably this is related to the effect of cooling on enzymes that are of importance for continuation of the aerobic metabolism; between 20 and 30°C there is a breakpoint, below which enzyme activity strongly declines (206). This may lead to a predominance of anaerobic metabolism and, consequently, to intracellular acidification and cellular degeneration.

The macroscopic appearance was, in general, not alarming. However, there appeared to exist a discrepancy with the light microscopical findings, caused by the fact that the latter refer to other parameters than macroscopic appearence.In the present study macroscopy reveals particularly gross necroses as BHN and circulatory disturbances as shown by a discoloration of all livers at the contact surface with their support. This is an argument for the development of liver supports in which the liver weight is more equally distributed during perfusion.

In conclusion, normothermic non flow anoxia and the "reperfusion phenomenon" play a primary role in the development of degenerative tissue alterations during normo- or hypothermic perfusion of the isolated rat liver, subjected to normothermic anoxia, whereas hypoxia as such primarily causes cell degeneration in normothermically perfused rat livers that are not subjected to anoxia. Only mild to moderate hypothermia probably exert litle protection against the effects of normothermic anoxia. Hypoxic alterations in not anoxia treated, normothermically perfused livers have to be regarded as a consequence of the application of an acellular perfusate.

CHAPTER 8

AMMONIA METABOLISM OF THE ISOLATED PERFUSED RAT LIVER, WHETHER OR NOT SUBJECTED TO NORMOTHERMIC NON FLOW ANOXIA

SUMMARY

Ammonia handling by the isolated perfused rat liver, whether or not treated with one hour normothermic anoxia prior to perfusion at 10,20,30 or 37°C for three hours, followed by a fourth hour of perfusion at 37°C, was studied.

Normothermic non flow anoxia did not influence ammonia metabolism adversely. Perfusion at 10 to 30°C nullified ammonia production, whereas rewarming and perfusion at 37°C increased ammonia release. This might be interpreted as a adverse effect.

INTRODUCTION

The liver plays a central role in the metabolism of ammonia, being involved in production as well as clearance of amonia. Production of ammonia involves two major steps: transamination followed by oxidative deamination, and oxidative deamination of aminoacids. Both reactions are located inside the mitochondria. The present study was undertaken to investigate the influence of cooling and anoxia on the handling of ammonia by the isolated rat liver, perfused with an aminoacid containing medium.

MATERIALS AND METHODS

The livers of 64 male Wistar outbred SPF rats, of which further characteristics were described in chapter 3, were used. Hepatectomy and perfusion was done as described in chapter 2. The perfusion protocol is outlined in chapter 3. The perfusate's content of aminoacids is given in table 8-1.

Table 8-1: Aminoacid content of Basal Medium Eagle (mg/l).

L-Arginine HCl	21.00
L-Cysteine 2HCl	15.65
L-Glutamine	292.00
L-Histidine	8.00
L-Isoleucine	26.00
L-Leucine	26.00
L-Lysine HCl	36.47
L-Methionine	7.50
L-Phenylalanine	16.50
L-Threonine	24.00
L-Tryptophane	4.00
L-Tyrosine	26.00
L-Valine	23.50

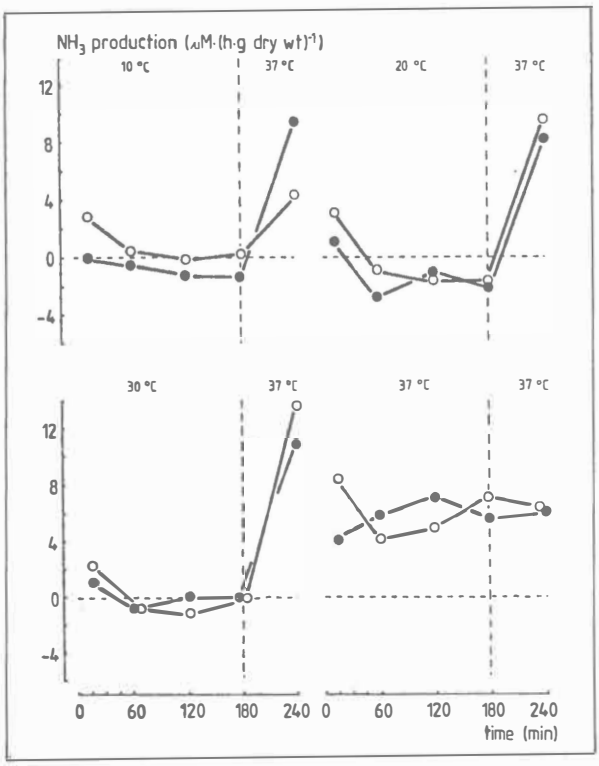


Fig.8-1: Median ammonia production (umol/h x g dry wt) of isolated rat livers whether or not subjected to one hour normothermic non-flow anoxia (O or● resp.) prior to perfusion at 10, 20, 30 or 37°C for three hours, followed by a fourth hour of perfusion at 37°C. There were no significant differences in ammonia production between the anoxia treated and the not treated livers (Wilcoxon's two sample test).n=8 for each group.

Samples of in- and outflowing perfusate were taken at 15, 60, 120, 180 and 240 minutes of perfusion. Ammonia concentrations were measured according to a modification of the trichloroaceticacid-direct-method (219). From these concentrations the ammonia production per hour per gram dry liver weight was calculated.

The urea concentration of the perfusate was measured enzymatically.

Comparisons were made between the livers during perfusion at 10, 20, 30 and 37 degrees centigrade, between anoxia treated and not

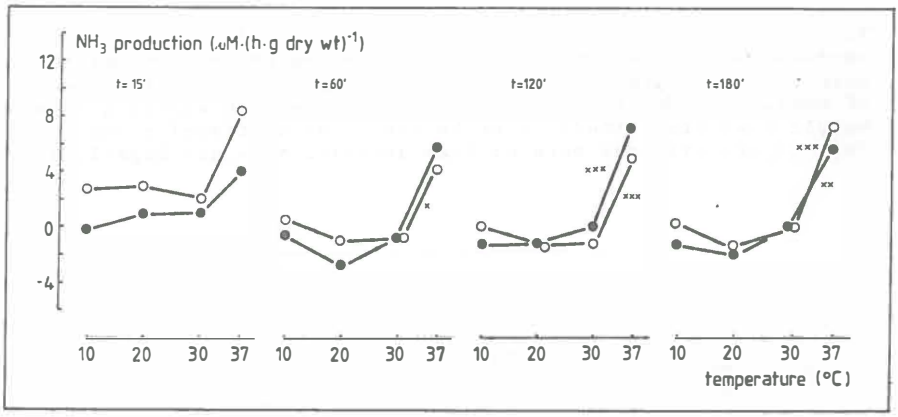


Fig.8-2: Median ammonia production (umol/h x g dry wt) of isolated perfused rat livers, whether or not (O or ●) subjected to one hour normothermic non-flow anoxia before perfusion after 15, 60, 120 and 180 minutes of perfusion. Differences in ammonia production of succeeding temperature groups were tested for significance with Wilcoxon's two sample test. * .05>P>.01; ** .01>P>.005; *** .005>P>.001.

anoxia treated livers, and between the livers after one hour of normothermic perfusion (t=240 minutes).

Statistical analysis was done as described in chapter 3.

RESULTS

During the first three hours of perfusion the hypothermically perfused livers produced less ammonia than the livers perfused at 37°C (Fig.8-1). This difference arose after 120 minutes for the non treated and after 60 minutes for the anoxia treated livers. After rewarming the ammonia production of the previously hypothermically perfused livers rose, and the differences disappeared (Fig. 8-2; Table 8-2).

Table 8-2 A and B: Differences in ammonia handling between livers perfused at 10, 20, 30 or 37°C, not treated (A) or treated (B) with one hour normothermic anoxia, during the first three hours of perfusion. Statistical analysis was done with Wilcoxon's two sample test (two sided). a or b= the first mentioned group in the left column produces more or less ammonia. ns = not significant.

		15'	60'	120'	180'
A	10 v 20	.08 b	ns	ns	ns
	20 v 30	ns	ns	ns	.02 b
	30 v 37	ns	ns	.001b	.008b
B	10 v 20	ns	ns	ns	ns
	20 v 30	ns	ns	ns	.06 b
	30 v 37	.08 b	.04 b	.004b	.004b

There were no differences in ammonia production between the anoxia treated and the not treated livers, before and after rewarming (Fig.8-1; Table 8-3).

After four hours of perfusion the not anoxia treated livers, previously perfused at 30°C, produced more ($P < .005$) than the livers, continuously perfused normothermically.Likewise the anoxia treated livers of the 10°C and 20°C groups produced more ammonia than the livers in the 30°C group. However, there was no difference between the 10 and 20°C groups at the one hand and the 37°C group at the other (Table 8-4).

The urea determination appeared to be too insensitive to obtain reliable results and to warrant any conclusion.

Table 8-3: Ammonia production (umol/h x g dry weight) of livers whether or not (II or I) subjected to one hour normothermic non-flow anoxia prior to perfusion at 10, 20, 30 or 37°C during three hours, followed by a fourth hour of perfusion at 37°C.The ammonia production was calculated from the ammonia concentration that was measured in samples of the in- and outflowing perfusate at 15, 60, 120, 180 and 240 minutes of perfusion. Statistical analysis was done according to Wilcoxon's two sample test (single sided). n=8 for each group. P= level of significance. Md =median. R= range. ns= not significant. T=temperature (°C).

		I		II		
T	/time	Md	R	Md	R	P
	15	.00	- 8.29 - 4.63	2.89	.58 - 11.46	.007
	60	- .50	-11.15 - 1.34	.58	-5.28 - 1.80	.08
10	120	-1.23	- 2.81 - 1.06	.00	-6.60 - .65	ns
	180	-1.26	-14.18 - 3.78	.28	-11.23 - 3.91	.07
37	240	9.45	-17.81 - 23.89	4.47	-5.76 - 11.78	ns
	15	1.07	-2.40 - 6.55	3.03	.00 - 4.62	ns
	60	-2.64	-2.57 - 1.02	- .92	-2.09 - 3.11	ns
20	120	-1.07	-1.71 - 1.88	-1.32	-2.85 - 1.80	.09
	180	-1.98	-2.57 - .00	-1.52	-3.20 - .56	ns
37	240	8.23	2.74 - 22.15	7.67	3.12 - 14.70	ns
	15	1.19	-.73 - 4.32	2.19	-.75 - 9.98	ns
	60	- .77	-2.74 - 3.38	-.77	-3.08 - 2.25	ns
30	120	.00	-7.61 - 1.63	-1.08	-4.95 - 3.15	ns
	180	.00	-2.13 - .88	.00	-1.61 - 3.60	ns
37	240	10.91	6.17 - 16.80	13.66	7.20 - 17.33	.1
	15	4.14	-16.80 - 9.14	8.36	-10.33 - 17.73	.05
	60	5.78	-10.11 - 12.91	4.22	-11.07 - 16.80	ns
37	120	7.12	2.50 - 19.01	5.00	.00 - 14.86	.10
	180	5.60	-.77 - 11.23	7.08	1.36 - 21.52	ns
37	240	6.01	2.16 - 9.98	6.36	.46 - 8.40	ns

Table 8-4: Levels of significance of the differences between the ammonia production of rat livers, whether or not (II or I) subjected to one hour normothermic anoxia prior to perfusion at 10, 20, 30 or 37°C for three hours, followed by a fourth hour of perfusion at 37°C. The data refer to the ammonia production after one hour normothermic perfusion. The level of significance was calculated according to Wilcoxon's two sample test (two sided).a or b = the first mentioned group in the left column produces more or less ammonia than the second group. n=8 in each group. ns=not significant.

	I	II
10 v 20	ns	ns
10 v 30	ns	.005 b
10 v 37	ns	ns
20 v 30	ns	.05 b
20 v 37	ns	ns
30 v 37	.005 a	.002 a

DISCUSSION

Under hypothermic conditions the production and the clearance of ammonia by the rat liver has shown to be nihil. The production of ammonia is related to the presence of aminoacids in the perfusate, since it has been shown that aminoacids may act as ammonia donors (220).It is, however, also imaginable that, when the liver is confronted with an overload of aminoacids, the ammonia liberation may exceed the capacity of the urea cycle.

There was no difference in ammonia handling between the anoxia treated and the not treated livers. Obviously, the ammonia metabolism is such a basic proces of the liver that it continues, even when the liver is damaged severely. The fact that ammonia handling remains undisturbed in damaged livers has been known for

many years (221), and was affirmed more recently (148). It should be noted, however, that these observations, done in an in vivo and an ex vivo perfusion model, primarily concerned the ammonia clearance. Probably the difference in observation between the latter two studies and the present one, is related to the type of perfusate used. It has been noted that ammonia liberation from glycine in rat liver perfusion experiments was lower when using diluted whole blood than when using an artificial medium, as in the present study (222).

Because there was no difference in ammonia handling between the anoxia treated and the not anoxia treated livers, it is tentative to conclude that the ammonia concentration of the perfusate does not reflect the severeness of liver injury, thus throwing a new light on the value of the ammonia concentration as a "liver function test" in the present setting.

As ammonia production by anoxia treated livers after four hours of perfusion, i.e. after rewarming, showed a tendency to be higher when the livers previously were perfused at a lower temperature, it might be speculated that hypothermia prevents or reduces damage to the deamination reactions inside the mitochondria, i.e. damage to mitochondria as such. However, these findings can also be interpreted in an other way since the liver is not only involved in the production, but also in the clearance of ammonia. When the clearance of ammonia is considered to be the principle function of the liver, production of ammonia can be seen as an adverse effect. From this point of view the results of the present study indicate that rewarming after previous hypothermic perfusion influences ammonia metabolism negatively.

Since the urea determination unfortunately appeared to be too insensitive, it is not possible to link the ammonia handling to the energy consuming urea production.

In sum, the following conclusions can be drawn: 1. Normothermic non flow anoxia has no adverse effect on ammonia metabolism of the liver. 2. Hypothermic perfusion at 30°C and below nullifies ammonia production by the liver, which may indicate that at these temperatures ammonia metabolism is preserved. 3. During normothermic perfusion the liver produces ammonia, which might be regarded as an adverse effect.

CHAPTER 9

CYTOPROTECTIVE EFFECT OF THE CALCIUM OVERLOAD BLOCKER FLUNARIZINE ON THE ISOLATED RAT LIVER SUBJECTED TO NORMOTHERMIC NON FLOW ANOXIA PRIOR TO HYPOTHERMIC PERFUSION: PRELIMINARY RESULTS

SUMMARY

The cell protective effect of the calcium overload blocker flunarizine (Janssen Farmaca, Belgium) was studied by perfusing 32 rat livers, half of which treated with flunarizine (flu) during flushing (500 ug per liver). All livers were subjected to normothermic non flow anoxie (60 minutes) prior to perfusion at 30 and 10°C for three hours, followed by a fourth hour of perfusion at 37°C.

Determinations comprized metabolic parameters (oxygen consumption, carbon dioxide production, respiratory quotient), perfusion characteristics, a synthetic parameter (bile production) and parameters for parenchymal integrity.

It appeared that flu exerts contradictory effects on isolated rat livers, subjected to normothermic non-flow anoxia: lower vascular resistance, depression of bile production and a higher release of ALT and AST. Therefore, in this model the cell protective effect of flu on anoxia treated livers remains inconclusive.

INTRODUCTION

A constant intracellular calcium concentration is of major importance in maintaining cellular integrity. Studies in intact animals and in cell cultures suggest that an ultimate influx of calcium ions accross injured plasma membranes and along steep concentration gradients, convert potentially reversible alterations into irreversible injury and cell death (223).
In liver transplantation and liver preservation the integrity of the liver cell is threatened by anoxia and hypoxia during donor hepatectomy, implantation of the liver, and possibly also during ischaemic preservation. Hypothermia has so far been shown to be the most effective means of reducing cellular injury during liver preservation.
In the knowledge that cellular calcium overload plays a central role in the cytotoxic mechanism which can be triggered by hypoxia or anoxia, prevention of calcium influx into the hepatocyte may possibly improve the outcome of liver preservation and transplantation, i.e. the quality of the liver that has to be transplanted.
In the present study, the effect of the calcium overload blocker flunarizine on rat livers, treated with normothermic non flow anoxia, was evaluated in the isolated perfused rat liver model.

MATERIALS AND METHODS

FLUNARIZINE

Flunarizine (Janssen Pharmaceutica, Beersse, Belgium), a difluorinated piperazine derivative (Fig. 9-1), is thought to

block the entry of calcium into cells selectively only in situations where calcium is stimulated to enter the cell in excess. It has been shown not to interfere with normal calcium homeostasis (224).

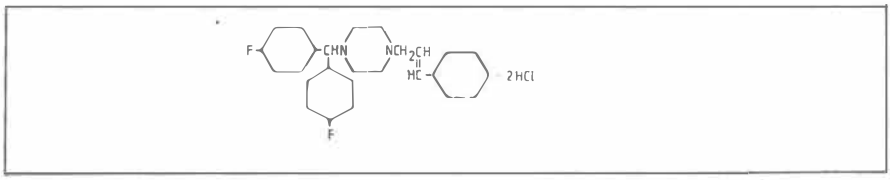


Fig.9-1: Structure of flunarizine hydrochloride.

In all probability flunarizine exerts its effect at the level of the plasma membrane. In vitro and in vivo studies show protection of endothelial cells against calcium overload, whereas flunarizine also has been shown to inhibit dose dependently membrane rigidity induced by calcium ion loading with a calcium ionophore in erythrocytes (225).
Metabolism of flunarizine in animals involves oxidative-N-dealkylation, aromatic hydroxylation and glucuronidation in the liver. In rats, biliary excretion accounted for 40 to 80 percent of a dose. In vivo only a neglectable amount is excreted unchanged in the urine (224).

EXPERIMENTAL SETUP

Thirty two outbred male Wistar SPF rats, weighing 290 to 435 grams were used. Hepatectomy and isolated rat liver perfusion were done as described in chapter 2.
All livers (n=32) were, before perfusion at 10 or 30°C, subjected to one hour normothermic non flow anoxia. The livers in

Table 9-1: The experimental protocol.

GROUP	n	FLUNA-RIZINE FLUSH	ANOXIA DURING 1 HOUR	3 HRS. PERF. AT ..°C	4TH H. PERF. AT 37°C
10F	8	yes	yes	10	yes
10C	8	no	yes	10	yes
30F	8	yes	yes	30	yes
30C	8	no	yes	30	yes

group 10F and 30F were, before the anoxic period started, flushed with 500 ml of perfusate containing a total of 0.5 mg flunarizine. Livers in group 10C (control) and 30C were flushed with 500 ml perfusate, lacking flunarizine. The four groups were 8 livers each.

To test the effect of the addition of flunarizine under normothermic conditions, all livers were rewarmed after three hours of hypothermic perfusion, and perfused at 37°C during a fourth hour (table 9-1).

MEASUREMENTS

Acid-base status, $\dot{V}_{O2}$, $\dot{V}_{CO2}$, R.Q., bile production, perfusion dynamics and dry/wet ratio were determined as described in chapter 3.

AST (GOT) and ALT (GPT) release were determined in the outflowing perfusate semi-automatically on an ACA apparatus (DuPont, USA). Since the protective effect of flunarizine should be most clear after rewarming, samples of the perfusate were drawn at 240 minutes of perfusion.

Statistical analysis was done as described in chapter 3.

RESULTS

Because of technical failures four livers had to be excluded from the study, leaving a total of 28 livers for evaluation. Also because of technical problems some determinations were unusable.

RESPIRATION

$\dot{V}_{O_2}$ of group 10C was significantly lower than in group 10F at 15 minutes. In the further course of the experiments there were no differences either between 10F and 10C nor between 30F and 30C (Fig. 9-2).

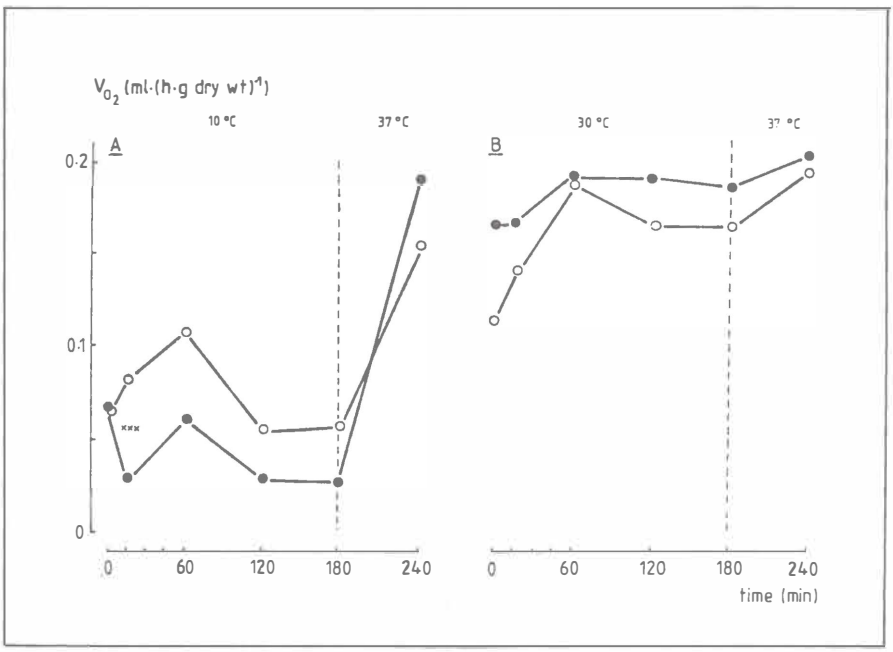


Fig.9-2 A/B: Median oxygen consumption (ml/min x gram dry weight) of livers perfused at 10 or 30°C, whether or not treated with flunarizine. ●=10°C, no flunarizine (n=8); O=10°C, flunarizine (n=6); ●=30°C, no flunarizine (n=8); O=30°C, flunarizine (n=6). Statistical analysis with Wilcoxon's two sample test. *** $P < .01$.

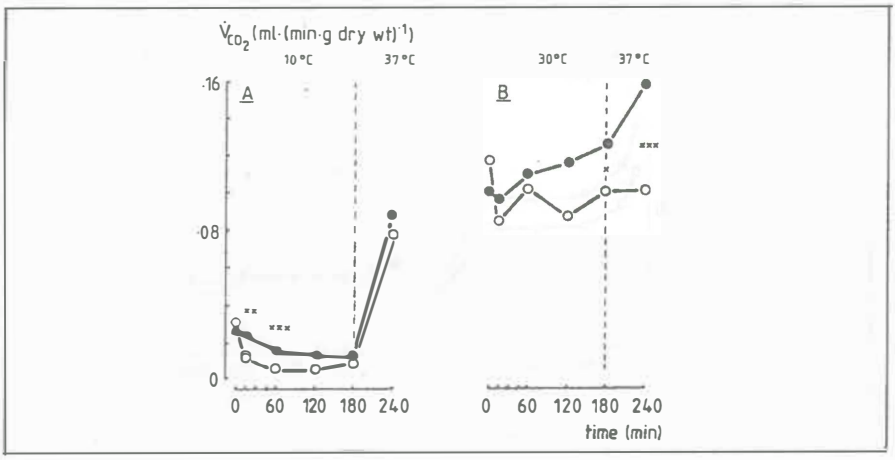


Fig. 9-3A/B: Median carbondioxide production (ml/min x gram dry weight) of livers perfused at 10 or 30°C, whether or not treated with flunarizine (flu). ●=10°C, no flu (n=8); O=10°C, flu (n=6); ●=30°C, no flu(n=8); O=30°C, flu (n=6). Statistical analysis with Wilcoxon's two sample test. * P < .05; ** P < .025; *** P < .01.

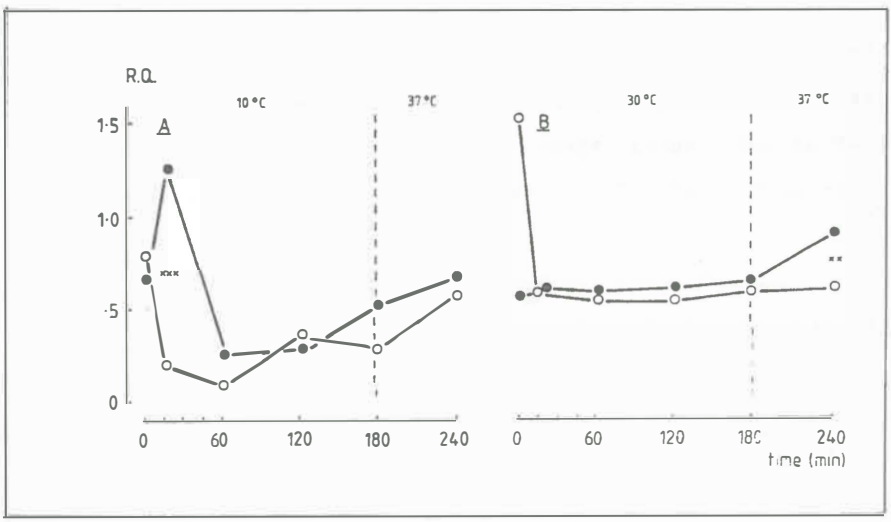


Fig. 9-4 A/B: Median R.Q. of livers perfused at 10 or 30°C, whether or not treated with flunarizine (flu). ●=10°C, no flu (n=8); O =10°C,flu (n=6); ●=30°C, no flu (n=8); O=30°C, flu (n=6). Statistical analysis with Wilcoxon's two sample test. ** P < .025; *** P < .01.

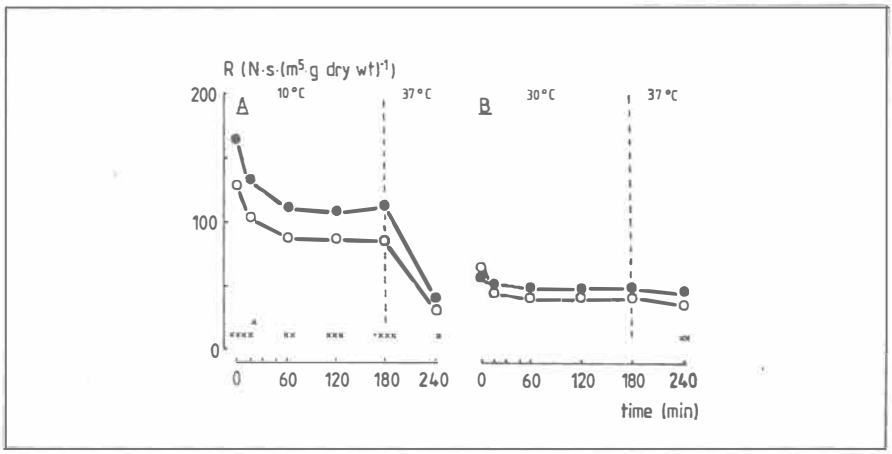


Fig. 9-5 A/B: Median vascular resistance (R; N.s/m^5 x gram dry weight) as calculated to the analogue law of Ohm of liver perfused at 10 or 30°C, whether or not treated with flunarizine (O and ● respectively. Statistical analysis with Wilcoxon's two sample test. * P < .10; ** P < .05; *** P < .025; **** P < .0 1.

The $\dot{V}_{CO2}$ was clearly lower in group 10F as compared to group 10C at 15 and 60 minutes. Group 30C, however, produced less CO_2 than 30F at 180 minutes. After rewarming there were no differences between 10F and 10C. 30F had then a lower CO_2 production than 30C (Fig. 9-3).

At 15 minutes group 10F had a lower RQ than group 10C. In the further course of perfusion there were no differences between these groups. After rewarming only group 30C had a lower R.Q. than 30F (Fig. 9-4).

PERFUSION DYNAMICS

Vascular resistance (R) of the livers in group 10F was lower throughout the experiments (fig.9-5A). Group 30C had only a lower R than group 30F at 240 minutes of perfusion (Fig. 9-5B).

BILE PRODUCTION

There were no differences in bile production between 10F and 10C (Fig.9-6A). Group 30F produced less bile during hypothermia at 150 and 180 minutes, and in normothermia at 210 and 240 minutes (Fig. 9-6B).

ENZYME RELEASE

After rewarming, groups 10F and 30F showed a higher AST(GOT) and ALT(GPT) ($P<.01$ and $P<.02$ resp.) release than 10C and 30C (Fig. 9-7). There were no differences during the hypothermic period.

DRY/WET RATIO

There were no differences in dry/wet ratio neither between groups 10F and 10C, nor between 30F and 30C.

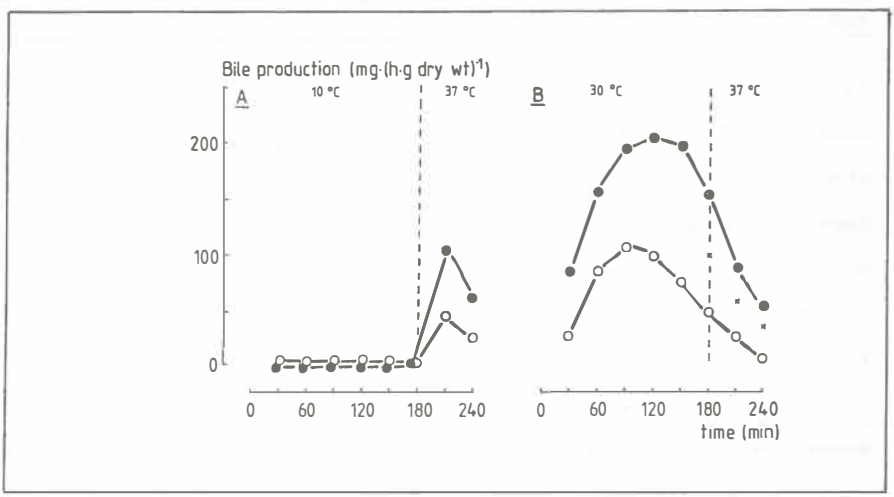


Fig. 9-6 A/B: Median bile production (mg/h x gram dry weight) of liver perfused at 10 or 30°C, whether or not pretreated with flunarizine (Flu). ● =10°C, no flu (n=8); O =10°C, flu (n=6); ●=30°C, no flu (n=8); O =30°C, flu (n= 6). Statistical analysis with Wilcoxon's two sample test. * $P < .05$; ** $P < .025$.

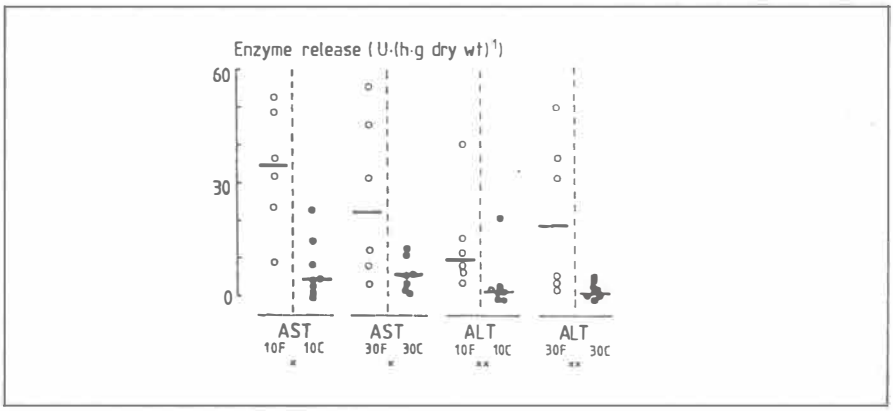


Fig.9-7: AST and ALT release (IU/h x g dry weight) of livers, whether or not pretreated with flunarizine (O or ●) after one hour normothermic perfusion (t=240 minutes), that was preceeded by three hours perfusion at 10 or 30°C. Statistical analysis with Wilcoxon's two sample test. * $P < .01$; ** $P < .02$.

DISCUSSION

Although it has been known for many years that cellular calcium overload plays a crucial role in cell injury and cell death, and although calcium overload and entry blockers were developed at least one decade ago, experience with these pharmaca in liver and organ preservation as a whole is scarce.

In the present study the influence of the calcium overload blocker flunarizine on anoxia treated rat livers was evaluated. So far, flunarizine had only shown to exert a cytoprotective effect on other cells than hepatocytes, among which endothelial cells and neurons (224).

The most remarkable and, at first sight, also paradoxal finding was the higher release of AST and ALT by flunarizine treated livers after rewarming. Although one may regard this as a

reflection of more serious tissue damage, it may also be speculated that livers, treated with flunarizine show less disturbances in their volume regulation, resulting in less formation of oedema and consequently less microcirculatory obstruction; i.e. flunarizine treated livers are more capable of releasing their enzymes. Hepatotoxicity has never been reported when using flunarizine concentrations as high as used in the present study. Besides, the difference in enzyme release was only seen after rewarming. This may indicate that at lower temperatures the microcirculation is constricted such that enzyme release is not possible, or that during rewarming hepatocytes are affected (by anoxia) leading to enzyme release and making it possible for flunarizine to exert its cyto-protective effects. Differences in bile production were only seen in livers, perfused at 30°C. This might be an effect of flunarizine of which it is known that it is excreted in bile and that it exerts its effect mainly on the level of the plasma membrane (224). It may be speculated that flunarizine interferes with the membrane permeability, thus reducing bile production. It is not likely that the pharmacon reduces membrane fluidity, since it has been shown to prevent the erythrocyte plasma membrane of becoming rigid under hypoxic conditions (224,226,227).

The lower vascular resistance (R) in the flunarizine treated livers, perfused at 10°C may be the result of the antivasospastic and oedema preventing properties of the drug. It is remarkable that this effect was most pronounced in profound hypothermia, when "normally" tissues are mostly apt to produce oedema as a result of disturbances in membrane bound cell volume

regulating mechanisms (186).

Since respiration parameters showed only minor differences, flunarizine obviously exerts no effect on the metabolic level. From the present study no definite conclusion can be drawn with regard to the cytoprotective effect of flunarizine on anoxia treated livers. With regard to organ, i.e. liver preservation, tne effect of calcium entry blockers should also be tested in a transplantation model. Recently some promising results were published on the effect of a calcium entry blocker on the outcome of kidney and liver preservation (228,229). In these publications, however, the calcium entry blocker was only given to the recipient.

Although these experimental setups are not wholy comparable to that of the present study they warrant further research on the possible role for calcium entry blockers in organ transplantation as well in improving the quality of the graft as in preventing damage during and after transplantation.

CHAPTER 10

EFFECT OF TAUROCHOLATE INFUSION ON BILE PRODUCTION OF THE ISOLATED PERFUSED RAT LIVER

SUMMARY

The effect of infusion of sodium-taurocholate and perfusate flow on the bile production of the isolated perfused rat liver was studied. Three livers were perfused normothermically during four hours. Taurocholate (0.025 umol/min) was infused during the last two hours. Determinations comprized bile production, perfusion characteristics and sodium- and potassium concentrations of the in- and outflowing perfusate.

Bile production declined during perfusion, and could not be stimulated to the initial level by infusion of taurocholate.

A relation between bile production and perfusate flow could not be brought about.

The ratio potassium to sodium increased during the experiments, possibly indicating deteoriation of membrane function.

Thus, reduction of bile production possibly reflects depletion of the bile acid pool as well as alterations at the level of the cell membrane, which may interfere with the cellular handling of bile acids.

INTRODUCTION

Bile production is a principle function of the liver, that can be used as an indicator of the performance of the isolated perfused organ. When perfused in an artificial perfusion system, the isolated liver immediately produces bile. It has been noted, however, that bile production decreases steadily in time. This might be caused by loss of the bile acids via bile drainage and/or a decrease in the syntheis of bile acids, secretion of which is thought to be a primary event in bile formation (231,232). Therefore, the aim of the present study was to investigate the influence of bile acid infusion on bile production by the isolated perfused rat liver.

MATERIALS AND METHODS

The livers of three Wistar outbred SPF rats, weighing 370 to 385 grams were used. The livers had a wet weight of 17.3 to 18.6 grams, as weighed before perfusion. After hepatectomy, which was performed as described in chapter 2, the liver was put in a recirculating perfusion system. Perfusion was done with 500 ml of a semisynthetic perfusate (chapter 2). Perfusion temperature was 37°C and perfusion pressure was kept constant (about 13 cm H_2O; 1.3 kPa).

The duration of each experiment was four hours. Only during the second two hours sodium taurocholate (0.025 umol/min; Calbiochem, La Jolla, USA) was infused.

Bile production was measured by collecting bile in preweighed plastic tubes every 15 minutes. From this the bile production per

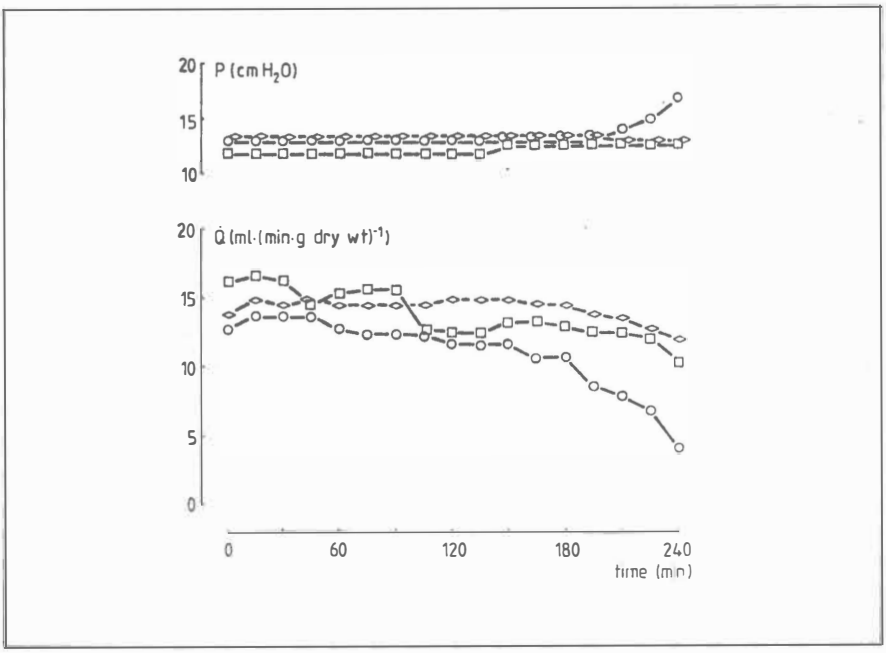


Fig.10-1: Perfusion characteristics. During the second two hours taurocholate was infused continuously. P=perfusion pressure (cm H_2O). Q=perfusate flow (ml/minx g dry wt).

hour per gram dry liver weight was calculated.

Perfusate flow was measured directly every 30 minutes (Fig. 10-1). To detect a possible relation between bile production and perfusate flow, these parameters were plotted against eachother.

The concentrations of K^+ and Na^+ in the perfusion medium were measured every 60 minutes. From this the ratio K^+ to Na^+ was calculated.

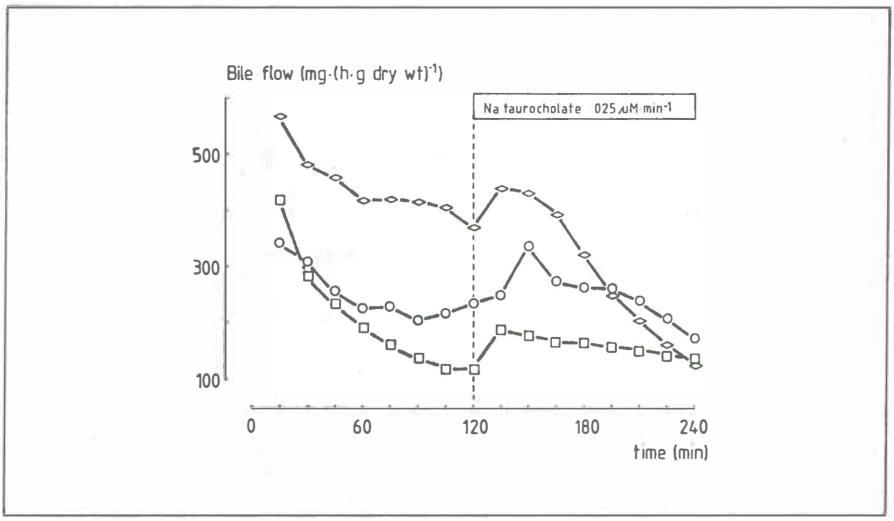


Fig.10-2: Bile production (mg/h x g dry wt) before and during infusion of sodium taurocholate (0.025 umol/min).

RESULTS AND DISCUSSION

Although infusion of taurocholate stimulated choleresis, bile production could not be stimulated to the initial level with this dose of taurocholate (Fig. 10-2). Throughout the first part of the experiments bile production declined, while only a small increase was observed just after the taurocholate infusion started in the second two hours of perfusion.

This leads to the conclusion that the decrease in bile production cannot only be explained by depletion of the bile acid pool. The decreasing perfusate flow throughout the experiments (Fig.10-1) potentially indicates one factor in the decrease of the bile production. However, the scatter in the plot of the bile prod ction against the perfusate flow was such, that regression-

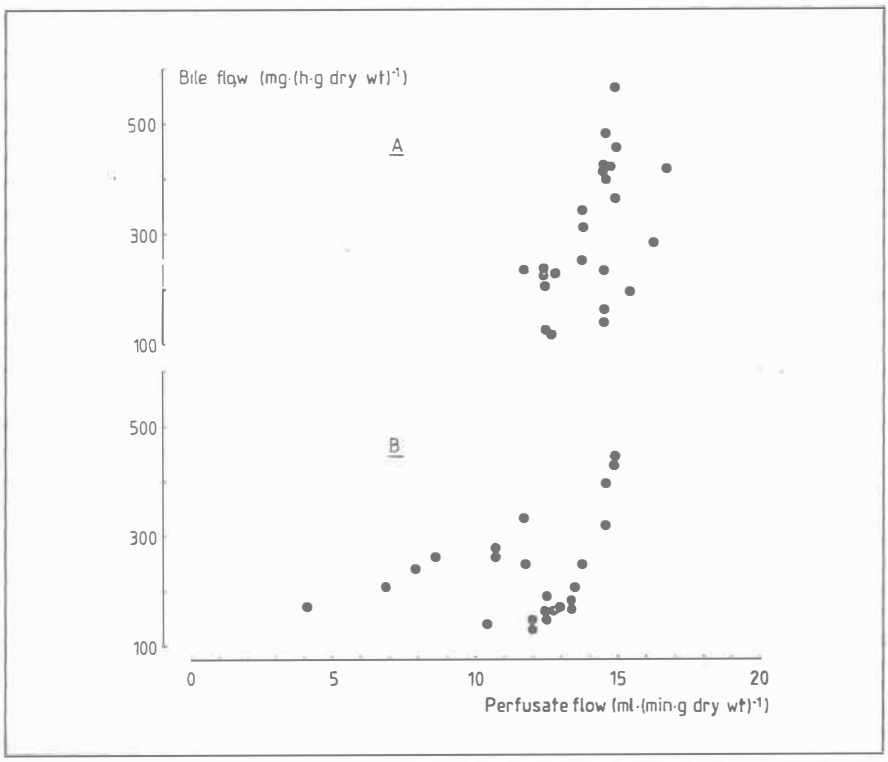


Fig.10-3: Perfusate flow plotted against bile flow. Bile flow increases with increasing perfusate flow, before (A) and during (B) infusion of taurocholate.

analysis was not allowed (Fig. 10-3).

Other causative factors in the reduction of the bile production may be alterations at the level of the cellular membrane or accumulation of toxic (cholestatic) metabolites.

In the present study the concentration of potassium increased relatively stronger than the concentration of sodium, as reflected in an increasing ratio of potasium to sodium (Fig.10-4).

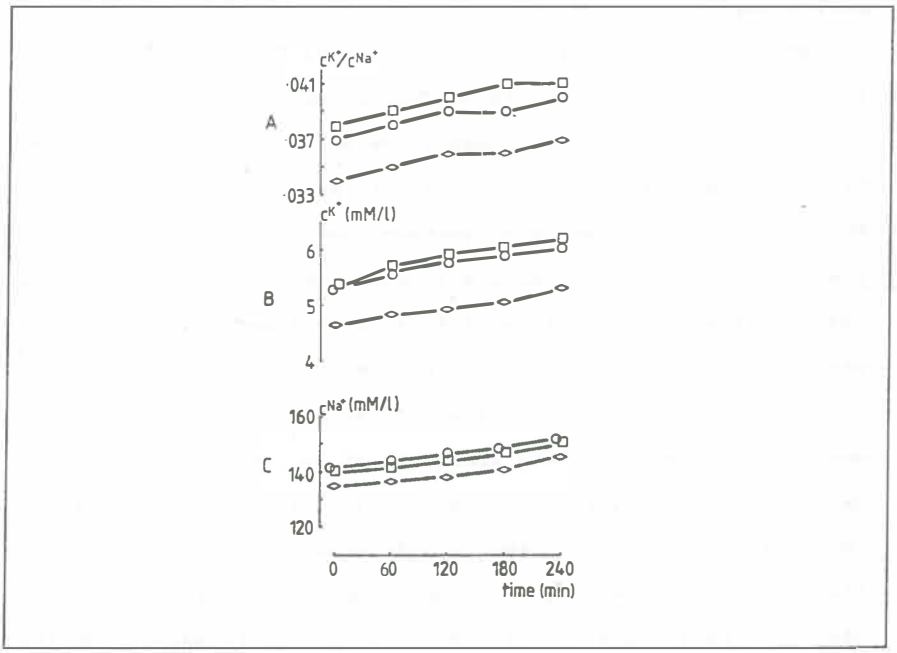


Fig.10-4: Potassiumconcentration of the perfusate (mmol/l; C) increased relatively stronger than sodium concentration (mM/l; B), as reflected in an increasing ratio potasium to sodium (A), during perfusion.

Obviously there was an increasing efflux of potasium from the cells when the perfusion experiments proceeded. This possibly reflects progressive deterioration of membrane bound energy dependend pump mechanisms or destruction of complete cells. In this respect it is possible that reduction of membrane bound Na/K-ATP-ase activity played a role, since a positive correlation has been found between such an activity in liver plasma membranes and bile production (233). Another factor could be the deficient oxygen delivery to zone 3 cells of the acinus and the resulting

cell damage.

In sum the following picture can be drawn: During perfusion of the isolated rat liver, bile production decreases, a phenomenon that cannot be reversed by continuous infusion of taurocholate. Simultaneously there is a reduction in perfusate flow. Progressive deterioration of membrane bound energy dependend pumps and diract cell damage are indicated in the perfusate's K^+-concentration. Continuation of bile production during isolated perfusion of the liver probably depends on preservation of energy dependent processes at the level of the plasma membrane.Diminution of the gradients of Na^+, K^+, Ca^{++} and Cl^- may cause swelling of hepatocytes and sinusoidal cells, leading to an increase in vascular resistance and reduction of the perfusate-flow. Deficient energy production or uncoupling processes may also lead to dissipation of the Na^+-gradient, necessary for the active uptake of taurocholate or active canalicular transport.

EPILOGUE

This thesis presents a model, which enables perfusion of the isolated rat liver in normo- and hypothermia. The model was characterized by using it in the study of function and morphology of the isolated rat liver, whether or not treated with normothermic anoxia prior to perfusion, under normo- and hypothermic conditions. The experimental studies focussed primarily on the effects of hypothermia and of anoxia on the isolated liver, and on the definition of parameters of viability of the liver during isolated perfusion.

THE MODEL

Isolated rat liver perfusion is one of many methods to study the functioning of an organ. Because an organ might be thought of as a coherent mass of cells, one can, when selecting a research model, apparently make a choice between isolated liver perfusion at the one hand, and models that applicate isolated cells and subcellular organelles at the other hand.That these methods of research are not equipotent is demonstrated in the present study. From the studies on the (physiological) functioning of the isolated rat liver, it can be concluded that 20°C is an ideal temperature to perfuse a liver at and, when taking liver transplantation into account, to preserve a liver at (chapter 4 to 6). However, the morphological studies (chapter 7) showed clearly that livers, perfused at 20°C and above had marked parenchymal damage. Thus, physiological appearance of the isolated liver was unimpaired, even when there was a major disturbance of the parenchymal architecture. Therefore, it must

be concluded that for research in liver preservation the isolated perfused (rat) liver model is the method of choice, to which data, gathered with the isolated cell model, may show to be complementary.

EFFECTS OF HYPOTHERMIA

Cooling exerts complex effects up on the functioning of the liver. These effects are not simply benificial (chapter 1). It has been shown that cooling reduces membrane fluidity, thus affecting transmembrane substrate transport and causing a reduction of energy metabolism. In the present studies these effects of cooling are reflected in a non linear relation between temperature at one hand and oxygen consumption, carbon dioxide production, respiratory quotient, lactate and pyruvate production and bile flow at the other hand(chapters 4,5,6). When these parameters are taken into account, the ideal temperature to preserve a liver at would be 20°C. This conclusion is in line with the finding that during the perfusion of the isolated rat liver at 20°C the ATP content, which is considered to be a crucial factor with respect to the outcome of preservation, remains constant over at least six hours (159).
It has, however, also to be noted that deep hypothermia at least minimizes anoxic parenchymal damage (chapter 7), whereas studies on the effect of hypothermia on pyruvate and lactate production as markers for aerobic and anaerobic glycolysis showed that anoxia treated livers were preserved best under moderate to profound hypothermic conditions (chapter 6). The same applies to

the production of ammonia (chapter 8).

EFFECTS OF ANOXIA

Several mechanisms may explain the occurrence of anoxia induced tissue injury. From the observations, described in chapter 7, it can be deduced that gross parenchymal damage does not arise during a period of anoxia but at the time of reperfusion with a well oxygenated perfusate. This reperfusion phenomenon has recently gained much attention. It is thought that the generation of oxygen free radicals, ultimately leading to a cellular calcium overload and deteoriation of the energy metabolism, plays a central role in its occurrence (213,214) (Fig. 1).

The unravelling of the mechanism of the reperfusion phenomenon

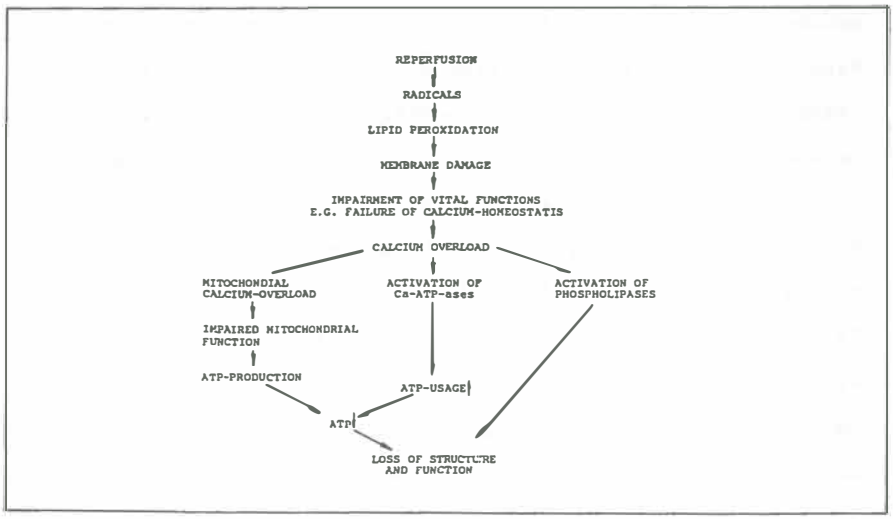


Fig.1: Pathways in the development of tissue damage by the "reperfusion phenomenon". After reperfusion, (oxygen) free radicals may cause membrane damage, thus disturbing calcium homeostasis. These events ultimately impair cellular structure and function.

opens possibilities for its prevention. In this respect the cytoprotective effect of the calcium overload blocker flunarizine was tested during hypothermic perfusion at 10 and 30°C (chapter 9). Although no final conclusions can be drawn from the data, it is clear that addition of this pharmacon reduced vascular resistance. This may eventually proof to be beneficial in long term preservation studies.

VIABILITY PARAMETERS

With regard to the definition of liver viability parameters during normo- and hypothermic perfusion of isolated livers, the present studies bring about only a limited amount of information. Only the assessment of bile production appeared to be a useful parameter (chapter 6). However, during perfusions of longer a duration (Appendix B) bile flow decreased gradually despite the infusion of bile acids, thus loosing its value as a viability parameter. At the other hand it was concluded in chapter 10, that this may be a reflection of metabolic deterioration of the hepatocyte. Therewith, the applicability of bile flow as marker for liver function is affirmed.
Taking multiple biopsies during liver preservation will, despite its specificity and sensitivity, prove to be less feasible because it is time consuming, is too laborious for the preservation setting and introduces the risk of haemorrhage after transplantation.

PRESERVATION TECHNOLOGY

The problem of the limited preservation time of livers is only partly a technical one, and concerns primarily the development of feed back circuitries for controlling the preservation process. Special attention should be payed to the positioning of the liver, because of its softness small vessels will be compressed under the liver's weight, causing pressure necrosis at the under surface when the liver is put on a solid support (chapter 7). Thus far, no optimal means of positioning the liver has been developed. It can be speculated that the optimal "preservation chamber" will mimic the situation in vivo (chapter 1).

As to the perfusion medium, a choice can be made between natural and artificial media. The perfusion model, presented in this thesis, demonstrates the applicability of a (modified) tissue culture medium as a perfusate. This finding is substantiated by previous reports (93).

The recent reports on the remarkable good results of simple cold storage of livers and other organs with the "UW"-solution (158) may warrant also further evaluation of this fluid in a recirculating system as the present model at higher temperatures at which the nucleotides in the medium may prove to be more effective.

It has been shown that high partial oxygen pressures are necessary to maintain aerobic energy transduction in hypothermia (4). Recently it has been shown that "hyperoxygenation" improves the ATP content of the liver cell (177), which was seen as a plea

for reintroducing hyperbaric oxygenation if it did not bring not so many extra costs with it. Another argument for at least reinvestigating the possibilities of hyperbaric oxygenation to counteract the effects of anoxia, is found in a study in which ischaemia damaged muscular tissue was shown to benefit from the introduction of hyperbaric oxygen (234).

FUTURE RESEARCH

It is clear that minimizing the detrimental effects of ischaemia, to which all livers are subjected during donor hepatectomy, presents a crucial first step in liver preservation. Therefore, one line of research
might deal with further elucidation of ischaemia induced tissue injury, thus enabling its prevention and/or counteraction. The use of newer research tools as NMR has already proved to be useful to this respect (159).
Another line of research may concern the understanding of the metabolic requirements of the isolated and cooled liver. For this purpose simple perfusion models, as presented in this thesis, are adventageous, although when the preservation of human livers is taken into account, perfusion of the livers of larger animals (e.g. dogs, pigs, primates) has to be considered.
Finally, requirements have to be formulated for methods that enable long term liver preservation. From the material presented in this thesis and the work of other investigators (85,127), it can be concluded that in particular perfusion preservation offers this possibility.

It is obvious that new developments regarding the construction and handling of the preservation equipment concerns primarily the mimicing of physiological regulation circuits. Consequently this requires the development of several cybernetic devices. Construction of the "hardware" will, presumably present relatively less problems in this era of automation. The crucial point, however, lays in the definition of "normal" for every temperature one wishes to preserve an isolated liver at. In this respect, the feasibility of the model for isolated rat liver perfusion, presented in this thesis, has been shown.

SUMMARY

This thesis concerns the development and application of a research model for normo- (37°C) and hypothermic (36°C and below) perfusion of the isolated rat liver. Three questions were put. Firstly, what is the effect of cooling on function and morphology of the isolated perfused rat liver. Secondly, what is the effect of cooling on function and morphology of the isolated rat liver, that is subjected to normothermic non flow anoxia. Thirdly, what are easily assesable variables, indicative for liver viability in normo- and hypothermia.

Chapter 1 reviews literature on hypothermia, preservation methods and preservation media.

The perfusion apparatus and the technique of hepatectomy in rat is described in chapter 2. The apparatus is suitable for double single pass and single recirculating perfusion under constant pressure. A tissue culture medium (Eagle's basal medium), enriched with insulin (20 IU/1), extra glucose (up to 10 mmol/1) and sodium bicarbonate (at least 17 meq/1) served as a perfusate.

In chapter 3 "materials and methods" with general application to the experiments, described in the following chapters, are presented.

Chapter 4 is concerned with the effect of cooling on lactate and pyruvate production and L/P ratio as markers of aerobic and anaerobic glycolysis.
Livers, perfused at 10 and 20°C produced less pyruvate and lactate than livers perfused at 30 and 37°C. The differences disappeared when all livers were perfused at 37°C. Median L/P

ratio was always about 6.
It was concluded that cooling depresses aerobic and anaerobic glycolysis reversibly.

Chapter 5 comprises the results of studies on the effect of cooling on several fundamental properties of the isolated rat liver.
The (calculated) vascular resistance (R) increased with cooling, possibly as consequence of the increasing viscosity of the perfusate. Bile production, oxygen consumption ($\dot{V}_{O2}$) and respiratory quotient (RQ) were lower in the 10 and 20°C groups. These differences disappeared after perfusion at 37°C.
The remarkable alterations in synthetic and metabolic activity between 30 and 20°C can be explained by changes in enzyme function and in membrane structure and function. In this, temperature induced changes in membrane "fluidity" are likely to play a central role.

In chapter 6 results are presented of studies on the effect of cooling on the functioning of isolated rat livers, that were or were not subjected to normothermic nonflow anoxia. Also the applicability of easily determinable variables ($\dot{V}_{O2}$, RQ, bile production, L/P ratio) as indicators of liver viability was studied.
Anoxia treated livers did not differ from the not treated livers regarding $\dot{V}_{O2}$, RQ and R. However, bile production of anoxia treated livers was significantly lower, which makes bile production an good viability parameter.
Pyruvate production of anoxia treated livers was lower during

continuous perfusion at 37°C. Anoxia treated livers produced only less lactate after rewarming after hypothermic perfusion. Obviously, hypothermic perfusion is necessary to prevent anoxic damage of aerobic glycolysis.

Mutual comparising of the groups of anoxia treated livers, led to the conclusion that, on physiological and biochemical grounds, 20°C was possibly an ideal temperature for perfusion or preservation.

Chapter 7 describes histopathologic alterations in livers, whether or not treated with anoxia. Light microscopically visible alterations appeared only when anoxia treated livers were (re-) perfused with a well oxygenated perfusate. In the occurrence of this "reperfusion phenomenon" the generation of oxygen free radicals and cellular calcium overload are apt to play a central role.
Perfusion at 10°C revealed, on histopathological grounds, better results than perfusion at 20°C or higher.

Ammonia metabolism of isolated rat livers that were or were not subjected to normothermic nonflow anoxia, was studied in chapter 8. Cooling reduced ammonia production strongly, whereas the livers produced ammonia under normothermic conditions. There were no differences between anoxia treated and not anoxia treated livers. Obviously ammonia metabolism continues even when the liver is injured severely.

Chapter 9 comprises the results of experiments on the effect of the calcium overload blocker flunarizine on the functioning of anoxia treated livers, perfused at 10 and 30°C.
Flunarizine treated livers showed a higher perfusate flow and a lower bile production, while they released more ALT and AST after rewarming to 37°C. It was concluded that flunarizine improves perfusion characteristics, which is reflected in the more pronounced liberation of intracellular enzymes, that are released after anoxic injury. The diminished bile flow is possibly related to the stabilizing effect of flunarizine on the plasma membrane.

Chapters 10 and appendix B describe the application of the perfusion apparatus as a single recirculating system. Appendix B concerns four medium term perfusion experiments, whereas chapter 10 deals with the question which mechanisms are involved in the decreasing bile production in perfusion experiments of longer duration. It appeared that depletion of bile acids as well as a decreasing perfusate flow played a role in the appearence of this phenomenon.

In the epilogue the data from the previous chapters are discussed and suggestions for further research are made.

SAMENVATTING

Dit proefschrift betreft de ontwikkeling en toepassing van een onderzoeksmodel voor de normo- en hypotherme perfusie van geisoleerde rattelevers in het kader van het onderzoek naar uitbreiding van de maximale preservatieduur van levers voor klinische transplantatie.

In het eerste deel (hoofdstuk 1) van het proefschrift wordt een overzicht gegeven van de literatuur over hypothermie, preservatietechnieken en preservatiemedia.
In het tweede deel (hoofdstuk 2) worden het ontwikkelde perfusiemodel en de techniek voor hepatectomie bij ratten beschreven.
In het derde deel van het proefschrift (hoofdstukken 3 t/m 11) worden de resultaten besproken van de experimentele studies over het functioneren van geisoleerde rattelevers onder normo- en (vooral) hypotherme omstandigheden. Daarbij staan de volgende vragen centraal:
1. Wat is de invloed van afkoeling op het functioneren en de morfologie van de geisoleerde rattelever.
2. Wat is de invloed van afkoeling op het functioneren en de morfologie van de geisoleerde rattelever, die is blootgesteld aan normotherme non flow anoxie.
3. Zijn er eenvoudig te bepalen variabelen, waarmee de vitaliteit van de geisoleerde rattelever onder normo- en hypotherme omstandigheden kan worden bepaald.

In hoofdstuk 1 wordt een overzicht gegeven van de literatuur over de invloed van afkoeling op fundamentele fysische, biochemische

en fysiologische processen in biologische systemen en op structuur en functie van biologische membranen en over preservatietechnologie.
Afkoeling heeft vooral invloed op de "fluidity" van biologische membranen; de "fluidity" neemt sterk af rond de 20°C. Dit heeft sterke invloed op het functioneren van de cellen en het energiemetabolisme.
In alle belangrijke levertransplantatiecentra worden levers momenteel gepreserveerd volgens de zogenaamde "simple cold storage" methode. Daarbij wordt de lever, na te zijn schoongespoeld met een elektrolietoplossing, op ijs bewaard. Continue perfusie van levers is tot nu toe weinig succesvol gebleken. Desalniettemin is het niet onwaarschijnlijk dat juist deze technieken de mogelijkheid zullen bieden de preservatieduur voor levers verder uit te breiden. Alleen met deze technieken is er namelijk continue aan- en afvoer van nutrienten en toxische metabolieten.
In de loop der tijden is een groot aantal natuurlijke (bloed, plasma, serum en afgeleiden) en kunstmatige (elektrolietoplossingen, weefselkweekmedia) media gebruikt voor de perfusie en preservatie van organen. De kunstmatige media worden in het algemeen geprefereerd vanwege hun constante samenstelling, het beperkte gevaar voor de overdracht van pathogene microorganismen en vanwege hun beschikbaarheid. Eenvoudige elektrolietoplossingen bevatten geen nutrienten. Deze moeten echter wel worden toegevoegd als de lever wordt geperfundeerd gedurende langere tijd en/of bij temperaturen boven 10°C, waardoor het metabolisme toeneemt.

Er is getracht de kwaliteit van de preservatiemedia te verbeteren door toevoeging van diverse stoffen om, onder andere, obstructie van de microcirculatie te voorkomen, het weefsel te beschermen en om het metabolisme te steunen of juist af te remmen.

Het perfusiemodel en de techniek van de hepatectomie bij de rat worden beschreven in hoofdstuk 2. Het ontwikkelde perfusieapparaat is afgeleid van bestaande systemen en is geschikt gemaakt voor dubbele "single pass" en enkelvoudige recirculerende perfusie onder constante perfusiedruk. Als perfusiemedium wordt een weefselkweekmedium (Eagle's basal medium) gebruikt, waaraan insuline (20 E/l), extra glucose (tot 10 mmol/l), albumine (2g/l) en natriumbicarbonaat (minimaal 17 mmol/l) worden toegevoegd.

In hoofdstuk 3 worden de "materials and methods" beschreven, voor zover die algemene geldigheid hebben voor de in de volgende hoofdstukken beschreven experimenten.

De in de hoofdstukken 4 en 6 beschreven experimenten betreffen het functioneren van de geisoleerde rattelever in normo- en hypothermie.
In hoofdstuk 4 gaat het om de invloed van afkoeling op de productie van pyruvaat en lactaat als "markers" voor aerobe en anaerobe glycolyse. Er wordt aangenomen, dat de productie van lactaat en pyruvaat onder normale omstandigheden in evenwicht zijn en de lactaat/pyruvaat ratio (L/P ratio) onder de tien blijft.
Levers, die bij 20 en 10°C werden geperfundeerd produceerden

minder pyruvaat en lactaat dan levers die bij 37 en 30°C werden geperfundeerd. Als de levers, die aanvankelijk hypotherm werden peperfundeerd, werden opgewarmd en geperfundeerd bij 37°C verdwenen deze verschillen. Tussen de 37 en 30°C groepen en de 20 en 10°C groepen bestonden geen verschillen.
De mediane L/P ratio was altijd ongeveer 6. Er werd geconcludeerd dat afkoeling de aerobe en anaerobe glycolyse reversibel remt.

De resultaten van de studies over de invloed van afkoeling op de perfusiekarakteristieken (perfusaatflow, Q_p ; vaatweerstand, R), celademhaling (zuurstofconsumptie, $\dot{V}_{O2}$; koolzuurproduktie $\dot{V}_{CO2}$; respiratoir quotient, RQ) en galproduktie zijn neergelegd in hoofdstuk 5.
De (berekende) R nam toe met het dalen van de temperatuur. Dit was waarschijnlijk het gevolg van de toegenomen viscositeit van het perfusaat in hypothermie.
De galproduktie, de $\dot{V}_{O2}$ en het RQ in de 10 en 20°C groepen enerzijds was lager dan in de 37 en 30°C groepen anderzijds. Er bestonden geen verschillen tussen de 20 en 10°C groepen en de 37 en 30°C groepen. Na opwarming verdwenen de gevonden verschillen. De opvallende veranderingen in metabole en synthetische activiteit tussen 30 en 20°C kunnen worden verklaard door veranderingen in enzymfunctie en membraanstructuur- en functie in dit temperatuurgebied. Hierin spelen veranderingen in membraan "fluidity" onder invloed van afkoeling een centrale rol.

In hoofdstuk 6 worden de resultaten beschreven van de studie, die erop was gericht na te gaan wat de invloed is van afkoeling op het functioneren van geisoleerde rattelevers, die geperfundeerd

worden na te zijn blootgesteld aan normotherme nonflow anoxie. Daarnaast werd gekeken of eenvoudig te bepalen variabelen ($\dot{V}_{O2}$, RQ, R, galproduktie, Lactaatproductie, pyruvaatproductie, L/P ratio) bruikbaar waren om de kwaliteit van levers tijdens normotherme en hypotherme perfusie na te gaan.
De anoxisch beschadigde levers toonden voor wat betreft de $\dot{V}_{O2}$, RQ en R eenzelfde beeld als de niet aan anoxie blootgestelde levers (hoofdstuk 5). De galproduktie aan anoxie blootgestelde levers was significant lager dan van de niet aan anoxie blootgestelde levers. Galproduktie is derhalve een goede parameter voor het functioneren, dan wel de kwaliteit van geisoleerde, geperfundeerde rattelevers in normo- en hypothermie. Met anoxie behandelde levers produceerden minder pyruvaat tijdens continue perfusie op 37°C, terwijl ze alleen minder lactaat produceerden na opwarming. De mediane L/P ratio was telkens ongeveer 6. Kennelijk is hypotherme perfusie noodzakelijk om beschadiging van de aerobe glycolyse te voorkomen.
Uit een onderlinge vergelijking van de verschillende groepen met anoxie behandelde levers bleek dat op grond van de bepaalde variabelen 20°C een ideale perfusie, c.q. preservatietemperatuur is.

De histopathologische veranderingen, die optraden in levers, die wel of niet werden blootgesteld aan normotherme nonflow anoxie, worden beschreven in hoofdstuk 7.
Lichtmicroscopisch waarneembare beschadiging lijkt pas op te treden nadat levers, die aan anoxie zijn blootgesteld, worden gereperfundeerd met een goed geoxygeneerd perfusiemedium. Bij het

ontstaan van dit zogenaamde "reperfusie fenomeen" spelen waarschijnlijk zowel het vrijkomen van vrije zuurstofradicalen als het optreden van een cellulaire calcium-overload een rol. Voorts bleek dat perfusie bij 10°C betere resultaten leverde dan perfusie bij 20°C of hoger.

Het ammoniak metabolisme van de geisoleerde rattelever, die wel of niet is blootgesteld aan normotherme nonflow anoxie, komt aan de orde in hoofdstuk 8. Hypothermie reduceerde de ammoniakproduktie sterk, terwijl de lever onder normotherme condities juist ammoniak produceerde. Daarbij was er geen verschil tussen wel of niet anoxisch behandelde levers. Kennelijk blijft het ammoniak metabolisme intakt, ook al is de lever ernstig beschadigd.

Aangezien cellulaire calcium-overload een belangrijke rol lijkt te spelen in het optreden van het zogenaamde "reperfusie fenomeen" en ischaemische weefselbeschadiging, werden experimenten gedaan waarbij anoxisch beschadigde levers, na wel of niet te zijn voorbehandeld met de calcium-overload blocker flunarizine (Sibelium®, Janssen farmaca), geperfundeerd bij 30 of 10°C. De resultaten hiervan zijn beschreven in hoofdstuk 9.
De met flunarizine behandelde levers hadden een hogere perfusaat flow en een lagere galproduktie, terwijl ze na opwarming tot 37°C meer AST en ALT vrijmaakten.
Flunarizine verbetert derhalve de perfusiekarakteristieken met als gevolg een beter uitwassen van bij celverval vrijkomende enzymen. De vermindering van de galflow hangt mogelijk samen met het stabiliserende effect dat flunarizine op de celmembraan

heeft.

In hoofdstuk 10 en appendix B wordt het in hoofdstuk 2 beschreven single pass perfusie apparaat toegepast als een recirculerend enkelvoudig systeem bij enkele "medium term" (8 tot 10 uren) perfusies (appendix B) en bij het beantwoorden van de vraag welk mechanisme aan het afnemen van de galproduktie bij langer durende perfusies ten grondslag ligt (hoofdstuk 10).
Bij twee van de vier middellange perfusie experimenten trad een opvallende afname van de perfusaatflow op, wellicht als uiting van metabole ontregeling. De parameters voor celademhaling ($\dot{V}_{O2}$, $\dot{V}_{CO2}$, RQ) bleven echter tamelijk constant. De galflow nam in het verloop van de experimenten altijd af.
De oorzaak hiervan kon niet, zoals wel gesteld is, alleen worden gezocht in uitputting van de galzoutpool. Er bleek tevens een significante correlatie te bestaan tussen galproductie en perfusaatflow.
In de epiloog worden de bevindingen uit de voorgaande hoofdstukken integrerend besproken en worden suggesties gedaan voor verder onderzoek.

APPENDICES

APPENDIX A

- Table I : Perfusate flow.
- Table II : Vascular resistance.
- Table III : Oxygen consumption.
- Table IV : Carbon dioxide production.
- Table V : Respiratory Quotient.
- Table VI : Bile production.
- Table VII : Pyruvate production.
- Table VIII: Lactate production.
- Table IX : Lactate/Pyruvate ratio.
- Table X : Dry/Wet ratio.

Table I: The perfusate flow (Q_p; ml/min x g dry weight; Median and Range) of rat livers not subjected (I) and subjected (II) to one hour normothermic non flow anoxia, perfused at 37 (A), 30 (B), 20 (C) and 10°C (D) for three hours. This period was followed by a fourth hour of perfusion at 37°C. n=8 (£ n=7). There were no significant differences between groups I and II at any temperature. Md=median; t=time (min).

		I		II		
	t	Md	Range	Md	Range	P
	0	7.48	5.00-12.30	6.04	3.33- 8.33	.1
	15	9.22	6.06-12.30	8.00	4.07- 9.56	.2
D	60	9.76	6.06-14.61	9.45	4.44-12.50	.9
	120	9.09	6.76-12.30	10.23	4.72-11.25	.4
	180	8.88	5.46-12.30	10.00	4.72-11.81	.07
	240	13.34	8.85-17.30	15.22	5.68-18.51	.9
	0	9.29	5.13-16.25	6.15	2.35-12.14	.3
	15	8.43	5.13-16.88	8.68	4.41-12.86	.07
C	60	9.08	8.26-15.63	10.34	4.71-12.14	.4
	120	9.89	7.83-15.63	10.82	5.00-12.90	.4
	180	9.68	7.83-15.63	10.66	4.71-12.50	.4
	240	11.10	8.70-13.13	12.21	5.29-15.00	.7 £
	0	11.99	8.33-17.28	10.31	3.44-15.00	.2
	15	11.99	8.95-14.80	11.69	8.75-14.58	.8
B	60	11.80	8.95-15.20	12.63	9.71-17.08	.4
	120	11.78	8.42-15.20	12.68	10.00-17.50	.6
	180	11.99	7.37-16.00	12.54	9.68-15.83	.9
	240	12.21	7.37-16.80	11.54	8.13-15.36	.6
	0	13.12	8.80-18.26	10.72	4.35-16.00	.2
	15	13.27	10.40-19.57	12.29	8.70-18.95	.3
A	60	14.12	12.96-19.52	13.19	11.54-21.05	.2
	120	13.82	12.22-20.87	13.04	10.38-20.00	.1
	180	13.41	12.69-20.87	12.40	10.87-18.42	.2
	240	12.92	10.74-15.65	11.45	8.46-14.00	.1

Table II: Vascular resistance (R; $Ns/(m \times 10^{5})$; Median, Range) of rat livers not subjected (I) or subjected (II) to one hour normothermic non flow anoxia. Livers were perfused for three hours at 37 (A), 30 (B), 20 (C) and 10°C (D), followed by a fourth hour of perfusion at 37°C. There were no significant differences between I and II.n=8 (£ n=7).

		I		II		
	t	Md	Range	Md	Range	P
	0	1.42	0.86-2.35	1.76	1.27-3.18	.07
	15	1.20	0.86-1.75	1.40	1.11-2.54	.1
D	60	1.16	0.72-1.75	1.19	0.85-2.39	.2
	120	1.30	0.86-1.59	1.14	0.94-2.24	.2
	180	1.26	0.86-1.94	1.17	0.90-2.24	.4
	240	0.84	0.61-1.33	0.73	0.64-1.82	.1*
	0	1.14	0.65-2.06	1.73	0.87-4.51	.09
	15	1.29	0.63-2.06	1.23	0.82-2.40	.9
C	60	1.22	0.68-1.28	1.05	0.87-2.25	.4
	120	1.13	0.68-1.35	0.98	0.89-2.12	.3
	180	1.12	0.68-1.35	0.99	0.85-2.25	.3
	240	0.98	0.81-1.23	0.91	0.71-2.00	.5
	0	.089	0.66-1.27	1.03	0.71-3.08	.2
	15	0.89	0.74-1.18	0.91	0.73-1.25	.8
B	60	0.90	0.75-1.18	0.84	0.62-1.21	.5
	120	0.90	0.75-1.26	0.84	0.61-0.53	.4
	180	0.88	0.74-1.44	0.85	0.67-1.18	.7
	240	0.84	0.70-1.44	0.85	0.30-1.30	.9
	0	0.81	0.64-1.20	1.04	0.63-2.43	.2
	15	0.81	0.60-1.07	0.91	0.56-1.22	.5
A	60	0.77	0.60-0.91	0.83	0.50-1.02	.5
	120	0.77	0.56-0.96	0.84	1.02-1.13	.2
	180	0.81	0.56-0.91	0.91	0.57-1.02	.09
	240	0.82	0.70-1.10	0.97	0.60-1.46	.2

Table III: Oxygen consumption (VO2; ml/min x g dry weight; Median, Range) of livers not subjected (I) and subjected (II) to one hour normothermic non flow anoxia, perfused for three hours at 37 (A), 30 (B), 20 (C) and 10°C (D). This period was followed by a fourth hour of perfusion at 37°C. Significant differences between I and II are seen only in group D after 60 and 120 minutes of perfusion. n was always 8. t=time (min); Md=median.

		I		II		
	t	Md	Range	Md	Range	P
	0	.055	.027-.112	.065	.034-.130	.7
	15	.042	.023-.101	.029	.010-.076	.4
D	60	.057	.041-.119	.062	.026-.118	.7
	120	.055	.037-.107	.030	.011-.128	.2
	180	.026	.023-.056	.028	.009-.053	.1
	240	.206	.125-.299	.191	.068-.295	.6
	0	.116	.101-.152	.090	.037-.149	.9
	15	.109	.073-.134	.105	.036-.134	.3
C	.60	.115	.090-.143	.110	.049-.133	.6
	120	.126	.097-.152	.109	.069-.150	.9
	180	.106	.084-.157	.106	.050-.130	.2
	240	.174	.124-.198	.197	.080-.239	.5
	0	.203	.117-.313	.165	.032-.248	.9
	15	.179	.172-.198	.166	.060-.229	.6
B	60	.205	.178-.223	.192	.105-.268	.7
	120	.203	.177-.228	.202	.107-.309	.7
	180	.231	.152-.256	.186	.118-.299	.2
	240	.240	.165-.287	.203	.132-.283	.3
	0	.214	.172-.341	.186	.044-.261	.9
	15	.249	.131-.314	.234	.089-.304	.6
A	60	.250	.206-.327	.225	.146-.339	.02
	120	.253	.182-.326	.190	.147-.361	.03
	180	.248	.199-.368	.221	.158-.291	.2
	240	.239	.183-.265	.169	.124-.244	.1

Table IV: Carbon dioxide production (VCO2; ml/min x g dry weight x 1000; Median, Range) of livers not subjected (I) and subjected (II) to one hour normothermic non flow anoxia, perfused at 37 (A), 30 (B), 20 (C) and 10°C (D) for three hours. This period was followed by a fourth hour of perfusion at 37°C. There were only significant differences between I and II at the beginning of perfusion at 10°C (A) and in the last two hours of perfusion at 37°C (D). n = 8 (*n=7). t=time (min); Md=median.

		I		II		
	t	Md	Range	Md	Range	P
	0	.010	* .001-.130	.136	* .015-.151	.05
	15	.012	* .004-.120	.116	* .012-.039	.04
D	60	.015	* .010-.170	.019	* .003-.022	.2
	120	.012	* .007-.170	.018	* .003-.021	.7
	180	.015	* .005-.080	.016	* .001-.017	.1
	240	.214	* .138-.280	.209	* .050-.259	.2
	0	.024	.010-.037	.036	* .004-.170	.8
	15	.026	.019-.049	.036	.009-.063	.3
C	60	.035	.016-.053	.032	.018-.048	.8
	120	.040	.016-.049	.031	.020-.043	.7
	180	.037	.018-.060	.031	.019-.065	.5
	240	.181	.115-.232	.132	.108-.217	.8
	0	.085	.005-.215	.052	.014-.234	.7
	15	.111	.068-.150	.097	.042-.132	.9
B	60	.133	.105-.181	.111	.042-.151	.9
	120	.140	.079-.198	.117	.050-.184	.2
	180	.160	.093-.231	.127	.062-.183	.9
	240	.255	.108-.329	.159	.104-.275	.3
	0	.133	.110-.209	.144	* .012-.307	.5
	15	.174	.083-.318	.174	.034-.269	.4
A	60	.205	.108-.299	.175	.110-.194	.5
	120	.194	.125-.281	.118	.063-.176	.01
	180	.205	.108-.261	.113	.062-.193	.01
	240	.167	.096-.253	.092	.044-.195	.01

Table V: Respiratory quotient (RQ; Median, Range) of livers not subjected (I) and subjected (II) to one hour normothermic non flow anoxia, perfused for three hours at 37 (A), 30 (B), 20 (C) and 10C (D), followed by one hour perfusion at 37°C. Most differences were not significant. n=8 (*n=7, +n=6, £n=5). t=time (min); Md=median.

		I		II		
	t	Md	Range	Md	Range	P
	0	0.17	+ 0.07-0.29	0.64	* 0.18-1.16	.04
	15	0.26	* 0.11-0.69	0.89	£ 0.16-1.31	.1
D	60	0.19	* 0.14-0.54	0.26	* 0.03-0.58	.2
	120	0.18	* 0.11-0.37	0.27	* 0.07-0.94	1.0
	180	0.39	* 0.17-0.65	0.50	* 0.04-1.56	.7
	240	1.08	* 0.71-1.27	0.64	* 0.36-0.97	.02
	0	0.19	0.08-0.34	0.48	* 0.04-1.42	.9
	15	0.24	0.19-0.67	0.42	0.17-0.81	.2
C	60	0.30	0.12-0.44	0.39	0.14-0.47	.2
	120	0.32	0.11-0.38	0.28	0.21-0.42	1.0
	180	0.35	0.16-0.56	0.28	0.24-0.59	.6
	240	1.08	0.58-1.40	0.90	0.63-1.30	.9
	0	0.54	0.23-0.79	0.55	0.44-1.34	.2
	15	0.61	0.38-0.85	0.57	0.41-0.89	.8
B	60	0.68	0.47-0.87	0.57	0.33-0.78	.5
	120	0.71	0.38-0.91	0.53	0.43-0.92	.2
	180	0.65	0.51-1.20	0.61	0.53-1.07	.8
	240	0.99	0.50-1.64	0.86	0.49-1.18	.8
	0	0.65	0.42-0.86	0.61	+ 0.19-0.85	.7
	15	0.81	0.40-1.30	0.77	0.38-1.13	.3
A	60	0.83	0.38-1.14	0.70	0.49-1.17	.9
	120	0.80	0.50-0.94	0.55	0.30-0.82	.1
	180	0.81	0.40-1.18	0.56	0.35-0.86	.1
	240	0.76	0.45-1.04	0.54	0.79-0.39	.01

Table VI: Bile production (mg/h x g dry weight) of livers that were not subjected (I) and livers that were subjected (II) to one hour normothermic non flow anoxia, perfused at 37, 30, 20 and 10 °C(groups A,B,C,D) during three hours, followed by a fourth hour of perfusion at 37°C. Livers subjected to anoxia produced generally less bile (Wilcoxon's two sample test). n=8 (£ n=7; + n=6). t=time(minutes). Md=median.

		I		II		
	t	Md	Range	Md	Range	P
	30	36.34	23.63- 66.00	1.57	0.00- 12.22	<.001
	60	34.84	23.63- 51.53	1.98	0.00- 9.44	<.001
	90	28.53	21.82- 50.00	2.45	0.00- 8.89	<.001
D	120	26.23	10.00- 35.00	2.91	0.00- 6.63	<.001
	150	27.26	9.09- 32.00	1.84	0.00- 6.63	<.001
	180	25.28	6.45- 38.67	1.16	0.00- 4.80	<.001
	210	350.83	284.24-430.67	104.73	38.50-238.18	<.001
	240	£337.00	71.25-438.00	61.94	15.20-293.57	.02
	30	98.20	72.80-131.53	13.34	3.53- 80.00	.001
	60	83.20	41.60-110.76	35.78	7.05- 93.57	.01
	90	97.61	60.00-118.20	50.96	12.35- 91.42	.003
C	120	98.56	60.00-123.42	55.65	18.23-100.00	.01
	150	97.55	60.00-121.71	61.93	20.00- 95.00	.02
	180	95.75	59.20-124.00	60.65	20.71- 95.71	.03
	210	£306.52	77.22-420.51	190.88	52.14-328.57	.2
	240	£252.29	87.22-425.12	147.85	25.00-382.58	.2
	30	£274.70	203.84-320.38	85.81	0.00-202.50	.001
	60	£302.28	245.26-328.68	156.87	2.28-368.57	.03
	90	£296.57	254.21-322.04	194.87	15.42-385.00	.02
B	120	£293.93	250.52-327.20	204.95	25.14-380.00	.03
	150	£294.28	251.57-324.80	199.69	31.42-275.35	.02
	180	£311.14	129.98-333.93	162.05	34.28-262.42	.03
	210	£284.24	87.77-471.20	89.34	13.13-231.61	.01
	240	£246.61	87.36-283.48	56.99	1.25-134.83	.003
	30	347.79	256.15-562.40	117.97	10.30-536.00	.01
	60	344.66	292.80-557.84	160.00	66.21-500.00	.03
	90	£370.74	333.20-522.88	120.97	52.12-432.80	.007
A	120	£345.40	274.92-507.68	90.81	24.21-368.00	.009
	150	+350.16	235.77-509.96	65.67	8.42-384.80	.01
	180	286.80	188.46-497.04	52.65	0.00-358.40	.007
	210	183.51	97.69-449.16	39.63	0.00-275.20	.007
	240	£145.29	70.47-373.92	28.13	6.08-161.60	.009

Table VII: Pyruvate production (mmol/h g dry weight) of livers not subjected (I) and subjected (II) to one hour normothermic non flow anoxia, perfused at 37, 30, 20 and 10°C (groups A, B, C and D resp.) for three hours, followed by a fourth hour of perfusion at 37°C. P=level of significance. Md=median. t=time (minutes). n=8; £n=7; +n=6.

		I		II		
	t	Md	Range	Md	Range	P
D	15	3.26	1.49- 22.11	1.10	0.53- 10.09	.08
	60	2.19	0.98- 9.12	1.15	0.24- 3.90	ns
	120	3.12	1.76- 9.97	1.42 +	0.00- 8.53	ns
	180	3.80	1.05- 6.77	0.97 £	0.00- 3.30	.01
	240	15.92	9.37- 33.13	6.87	2.25- 18.36	ns
C	15	3.44	1.52- 8.75	1.85 £	0.00- 4.66	.01
	60	2.74	0.56- 11.90	2.10	0.22- 4.81	ns
	120	10.20	2.47- 26.85	6.94	1.09- 21.74	ns
	180	5.85	1.50- 23.64	3.32	0.38- 13.18	ns
	240	30.63	15.05- 40.94	15.11	3.75- 31.25	ns
B	15	20.22	9.93- 43.72	9.40	3.30- 26.08	ns
	60	21.46	8.44- 41.58	14.03	7.43- 29.97	ns
	120	22.20	19.17- 32.92	18.92	14.99- 42.30	ns
	180	33.87	17.68- 55.09	22.74	15.12- 27.62	.1
	240	33.90	17.07- 52.42	19.00	7.21- 55.10	ns
A	15	35.55	1.60- 38.13	11.10 £	0.96- 20.24	.01
	60	27.35	8.63- 38.05	14.44	0.62- 23.62	.01
	120	40.60	12.70- 65.42	15.31	9.12- 38.98	.01
	180	41.27	16.88- 77.28	9.31	6.85- 39.60	.01
	240	43.43	7.15- 63.00	12.53	3.50- 36.01	.01

Table VIII : Lactate production (mmol/h g dry weight) of livers not subjected (I) or subjected (II) to one hour normothermic non flow anoxia, perfused at 37, 30, 20 or 10°C (groups A, B, C and D resp.) for three hours, followed by a fourth hour of perfusion at 37°C. Md=median. t=time(minutes). P=level of significance. ns=not significant. n=8.

		I		II		
	t	Md	Range	Md	Range	P
	15	25.83	9.24- 49.43	35.46	15.00-111.00	ns
	60	23.76	6.15- 63.91	19.93	2.48- 73.58	ns
D	120	22.98	8.64- 39.02	22.77	11.06- 46.04	.10
	180	24.97	13.22- 49.38	20.63	14.46- 40.04	ns
	240	150.78	82.47-190.50	78.92	30.27-105.50	.01
	15	30.82	19.34- 40.42	43.16	9.93- 64.14	ns
	60	22.13	10.56- 49.02	29.34	3.48- 57.56	ns
C	120	41.68	21.46- 78.55	31.28	6.31- 79.66	ns
	180	39.16	8.48- 69.54	41.99	6.70- 63.68	ns
	240	132.61	64.73-259.51	105.31	73.71-272.30	.04
	15	104.19	66.08-267.84	85.35	55.12-124.68	ns
	60	115.85	85.25-200.20	131.75	16.38-212.12	ns
B	120	134.15	73.60-157.78	105.71	56.10-201.11	ns
	180	112.89	61.06-161.92	118.47	66.78-164.88	ns
	240	222.49	127.02-303.61	127.71	63.96-339.83	.02
	15	203.51	51.98-319.73	156.75	37.20-339.25	ns
	60	158.33	109.77-211.69	115.52	70.85-177.57	ns
A	120	173.11	79.94-257.54	105.83	67.80-146.64	ns
	180	161.58	59.42-324.94	78.05	41.82-124.65	ns
	240	138.06	96.99-178.67	51.88	33.81-119.81	.01

Table IX: Lactate/Pyruvate ratio of livers, not subjected (I) or subjected (II) to one hour normothermic non flow anoxia before perfusion at 37, 30, 20 or 10°C for three hours (groups A,B,C,D),followed by a fourth hour of perfusion at 37°C. Md=median. P=level of significance. t=time(minutes). n=8; £n=7; +n=6.

		I		II		
	t	Md	Range	Md	Range	P
	15	5.84	2.24- 23.36	25.98	5.92- 82.35	ns
	60	9.18	3.15- 24.64	16.72	1.38- 82.21	ns
D	120	6.67	2.93- 13.27	10.48+	2.72- 80.97	ns
	180	10.98	1.99- 17.68	26.69£	5.88-169.64	ns
	240	9.31	5.05- 19.08	7.82	5.46- 23.98	ns
	15	7.27	3.97- 20.31	21.68£	11.43- 46.21	.02
	60	6.18	3.49- 35.66	15.07	3.58- 77.28	ns
C	120	4.24	0.93- 29.66	5.52	0.57- 17.08	ns
	180	4.30	1.45- 20.50	12.42	2.93- 20.81	ns
	240	5.64	2.15- 8.47	10.47	3.04- 30.80	ns
	15	4.52	2.90- 11.45	9.38	4.58- 22.92	.01
	60	5.06	2.96- 15.60	7.07	1.82- 28.55	ns
B	120	5.00	3.58- 6.78	5.48	3.46- 7.97	ns
	180	3.59	1.68- 6.05	4.72	4.06- 8.23	.1
	240	6.10	2.81- 9.52	9.11	3.30- 15.66	ns
	15	7.62£	1.36- 9.86	9.29£	5.40- 29.26	ns
	60	6.24	3.70- 16.23	8.52	4.93- 23.04	ns
A	120	5.09	2.79- 8.45	5.78	3.28- 11.84	ns
	180	4.14	3.03- 5.77	5.49	2.67- 14.19	ns
	240	3.58	2.14- 13.57	4.70	3.24- 16.04	ns

Table X: Dry/Wet ratio of livers whether or not (II or I) subjected to one hour normothermic non flow anoxia prior to perfusion at 37,30,20 or 10°C (groups A,B,C,D), followed by a fourth hour perfusion at 37°C. Md=median. t=time (minutes). n=8. P=level of significance.

		I		II		
	t	Md	Range	Md	Range	P
D	240	0.20	0.15- 0.21	0.17	0.16- 0.19	ns
C	240	0.19	0.12- 0.21	0.18	0.14- 0.23	ns
B	240	0.16	0.13- 0.19	0.19	0.16- 0.22	ns
A	240	0.16	0.13- 0.18	0.14	0.10- 0.19	ns

APPENDIX B

MEDIUM TERM RECIRCULATING HYPOTHERMIC PERFUSION OF THE ISOLATED RAT LIVER; SOME ADDITIONAL EXPERIMENTS

SUMMARY

Some additional experiments on the medium term (maximally 10 hours) recirculating perfusion of four isolated rat livers are presented.

After six hours of perfusion the perfusate flow of two of the livers showed a steadily decline. Oxygen consumption, carbon dioxide production and respiratory quotient remained constant. Bile production of all livers declined throughout the experiments.

Possible explanations for these phenomena are discussed.

INTRODUCTION

Some additional findings are reported from medium term (eight to ten hours) perfusion experiments performed at 20°C.

The single pass perfusion model, outlined in chapter 2, is not suitable for medium and long term perfusion/preservation because too large volumes of perfusate will be needed, which is impracticable in particular in the clinical setting.

The set up of the recirculating perfusion system is schematically outlined in figure 1.

The observations, presented here concern solely perfusions at 20°C, because in previous experiments (chapters 4 to 6) this temperature might, on physiological grounds, be of interest for future liver preservation methods.

This report is descriptive in nature. Therefore no statistical analysis was done.

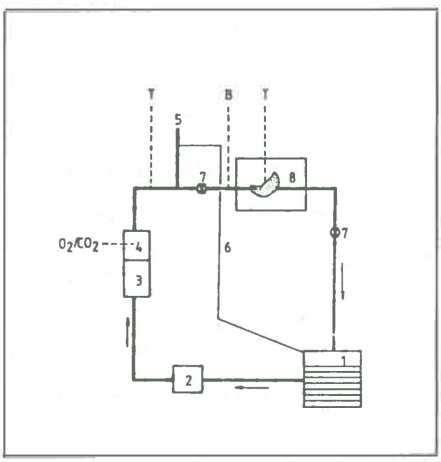


Fig.1: Schematic outline of the recirculating perfusion system: 1.perfusate reservoir, placed on an electromagnetic stirrer. 2. roller pump. 3. heatexchanger. 4. membrane oxygenator. 5. overflow system, enabling pressure adjustment. 6. backflow system from overflow to perfusate reservoir. 7.threeway stopcocks. T temperature probes. B infusion system for bile acids.

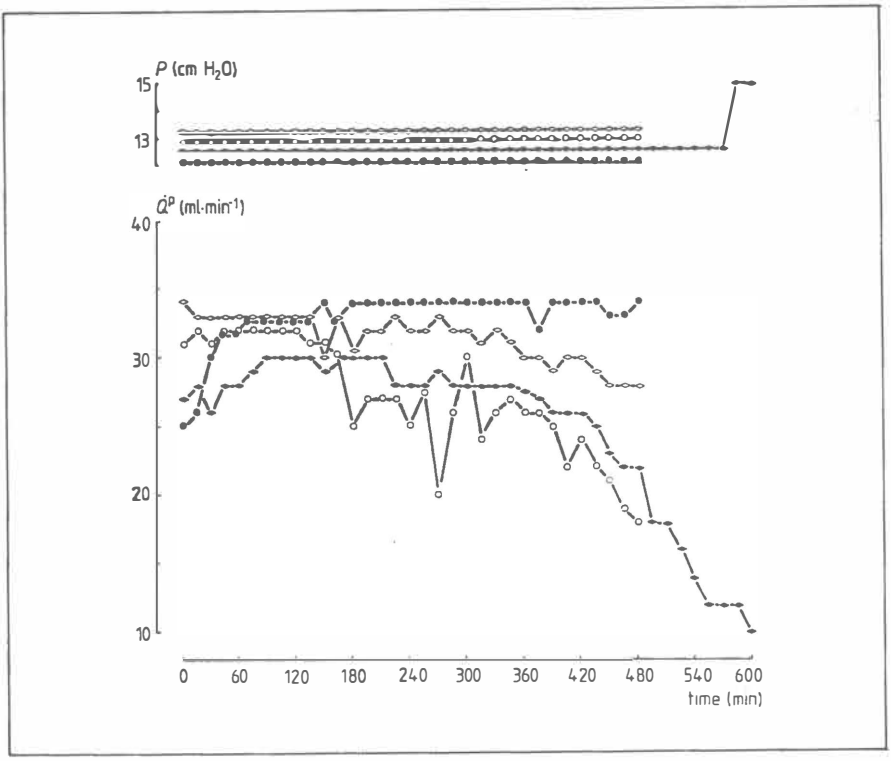


Fig.2: Perfusion pressure (cm H2O) and perfusate flow (ml/min).

PERFUSION DYNAMICS

Perfusion pressure was kept constant throughout the experiments. Only in the ten-hour experiment it was necessary to raise the pressure towards the end, because the perfusate flow decreased (Fig.2).

Perfusate flow remained fairly constant up to six hours of perfusion. Then two livers showed a steady decline in perfusate flow, which continued till the end of the experiment. The other

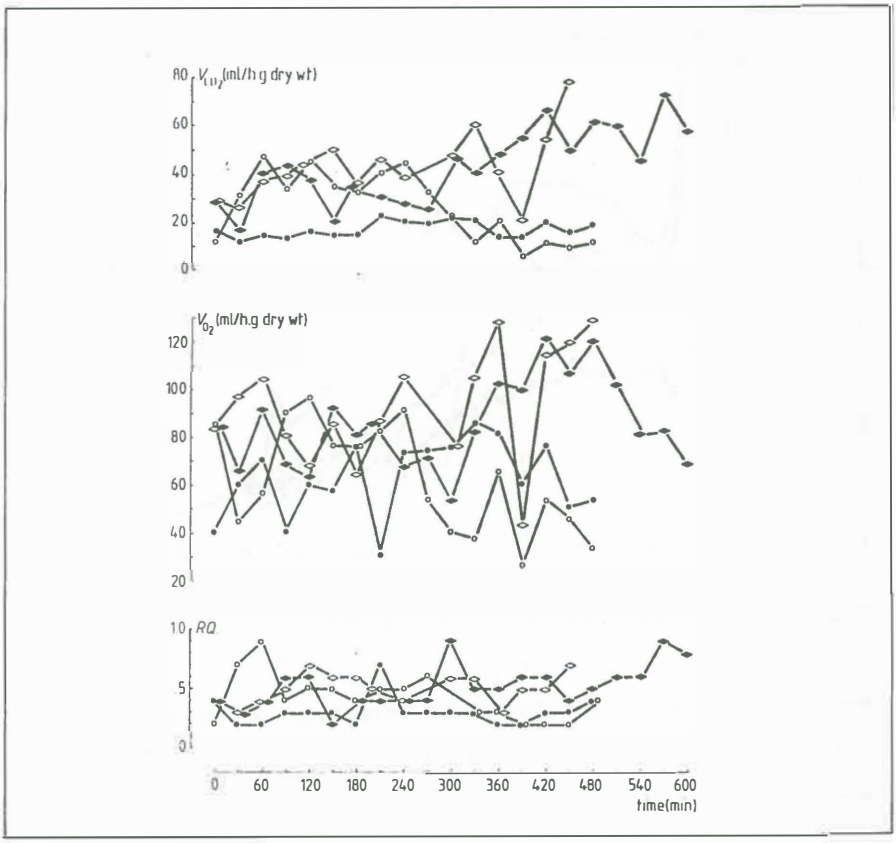


Fig.3: Oxygen consumption (ml/h x g dry wt x 1000), carbon dioxide production (ml/h x g dry wt x 1000) and respiratory quotient (= carbon dioxide production/oxygen consumption).

two livers showed a constant flow throughout the experiment. The phenomenon of the fall in perfusate flow has been known for many years in perfusion preservation, and has been linked to many factors (6). Numerous attempts have been made to prevent or to counteract this event (chapter 1). The present study shows, however, that it is also possible, without taking additional

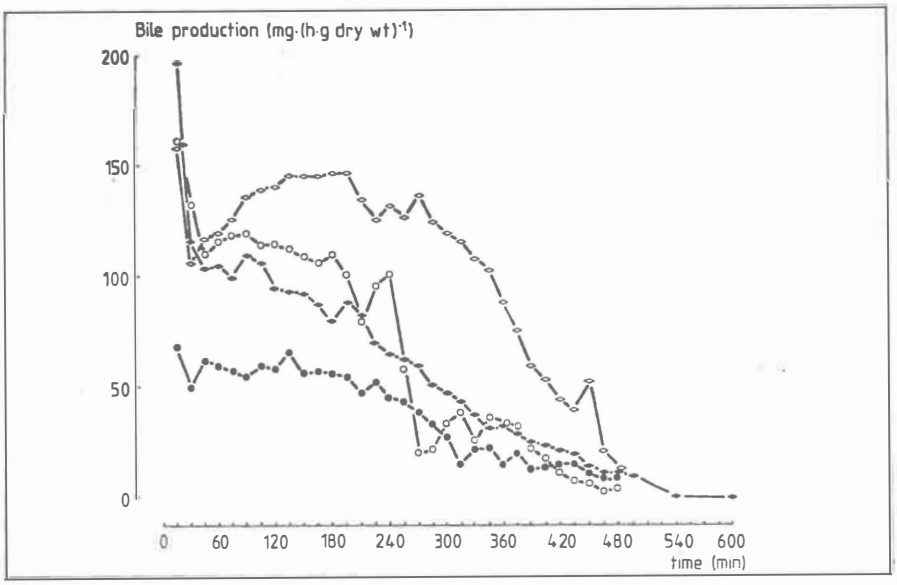


Fig.4: Bile production (mg/h x g dry wt).

measures, to keep a constant perfusate flow. Therefore, future research might be aimed at solving the question why one liver does not and the other does show obstruction of perfusate flow when the perfusion conditions are presumably entirely comparable. A possible explanation might be found in deterioration of the energy metabolism. During the preservation period, there is a progressive loss of adenine nucleotides (230). In this respect it has been shown that the ATP content of the liver can remain stable during perfusion at 20°C up to 6 hours (159). This is in line with the findings on two of the four livers.

METABOLISM

Oxygen consumption, carbon dioxide production and respiratory quotient remained constant throughout the experiments (Fig. 3). This is, however,no reflection of the condition of the liver, since there have been shown in a previous studie (chapter 6) to exist no differences in these parameters between anoxia damaged and undamaged livers.

BILE PRODUCTION

Bile production decreased in all livers throughout the experiments (Fig. 4). It has been put forward that this is related to exhaustion of the bile acid pool (231). However, in the present experiments a constant infusion of bile acids was given. Therefore it is tentative to conclude that there must be more causative factors beside bile acid depletion.

APPENDIX C

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DANKWOORD

Velen hebben op enigerlei wijze bijgedragen aan de totstandkoming van mijn proefschrift.

Mijn bijzondere dank en waardering gaat uit naar mijn beide promotoren.

Prof. Dr. C.H. Gips dank ik voor de stimulerende en persoonlijke wijze waarop hij de "groei" van dit proefschrift begeleidde.

Ik dank Prof. Dr. R. van Schilfgaarde voor de manier, waarop hij de superviserende taak van Prof. Dr. R.A.F. Krom, die het aan dit proefschrift ten grondslag liggende onderzoek opzette, voordat hij naar de Verenigde Staten vertrok, overnam. Als tweede promotor wist hij het werk, dat reeds voor een groot deel was verricht, mede te structureren, opdat het zijn huidige vorm kon krijgen.

Prof. Dr. R.A.F. Krom dank ik voor de wijze waarop hij mij betrok bij het onderzoek naar nieuwe methoden voor leverpreservatie. Hij liet mij veel vrijheid om eigen ideeen aangaande de onderzoeksopzet te verwezenlijken.

Prof. Dr. H.J. Houthoff was van begin af aan betrokken bij het onderzoek. Ik dank hem voor de beoordeling van alle weefselcoupes en zijn supervisie bij het schrijven van hoofdstuk 7.
De gesprekken, die ik met hem had, vooral ook over onderwerpen die buiten de "scope" van het onderzoek lagen, waren buitengewoon leerzaam.

Voor diverse delen van het onderzoek werd voorts advies gegeven door Dr. P. Rispens, Dr. J. Wilschut, Dr. R. Berger, Dr. R.J. Vonk en Prof.Dr. Ch.R.H. Wildevuur. Mijn dank hiervoor.

Prof. Dr. M.J. Hardonk, Prof. Dr. D.K.F. Meijer en Prof. Dr. G.L. Scherphof dank ik voor de manier waarop zij het manuscript van hun opbouwende kritiek voorzagen.

De medewerkers van het Laboratorium voor Hepatochemie (Ineke Pieters, Eli Nordeide, Petra Hoving) deden een groot aantal bepalingen in het kader van de experimenten. In het bijzonder dank ik Reint Huizenga, hoofd van het laboratorium, voor de vriendschappelijke samenwerking en de constructieve manier, waarop hij telkens meedacht bij het opzetten van nieuwe experimenten.

Zonder de aanwezigheid van biotechnicus Pieter Klok, waren de leverperfusie-experimenten ongetwijfeld een moeizame en eenzame onderneming geworden.
Ook de medewerking van de "keuzeco's" Wout Floris en Paul Metternich, die aan een groot aantal experimenten hun medewerking verleenden en bijdroegen aan de totstandkoming van hoofdstuk 9, was in dit verband van grote waarde.
De medewerkers van het Centraal Klinisch Chemisch Laboratorium (Eddy Ligeon, Greetje Sienot) verrichtten diverse bepalingen. Ook hen allen dank ik voor hun bijdrage aan mijn proefschrift.

Echter, zonder het geduld, dat Jolien, Julie en Sarah opbrachten, opdat ik mij zo lang en intensief bezig kon houden met het schrijven van een boekje, waarvan het onderwerp zo ver buiten de dagelijkse aktualiteit ligt, was mijn proefschrift er nooit gekomen. Vandaar dat ik mijn proefschrift aan hen opdraag.

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